Dissecting phosphate signalling in *Arabidopsis*

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ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylic acid
ADP	adenosine diphosphate
AT	after transfer
ATP	adensosine triphosphate
AMP	adenosine monophosphate
ARR	auxin response regulator
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
C	carbon
CaMV	Cauliflower Mosaic Virus
CDK	cyclin dependent kinase
cDNA	complementary DNA
CHA	chorismate
Col0	Columbia 0 ecotype (wild type)
DAHP	3-deoxy-Darabino-heptulosonate-7-phosphate
DEPC	diethyl pyrocarbonate
DHQ	dehydroquinate
DNA	deoxyribonucleic acid
1,3-DPGA	1,3-diphosphoglycerate
E4P	erythrose 4-phosphate
EPSP	5-enolpyruvylshikimate 3-phosphate
ER	endoplasmic reticulum
Fru-6-P	fructose 6-phosphate
Fru-1,6-P2	fructose 1,6-bisphosphate
GFP	green fluorescent protein
GUS	β-glucuronidase
Glu-1-P	glucose 1-phosphate
Glu-6-P	glucose 6-phosphate
G3P	glyceraldehyde 3-phosphate
G3PDH	glyceraldehyde 3-phosphate dehydrogenase
IAA	indole-3-acetic acid
KIN	kinetin
LB	Luria-Bertrani medium
LUC	luciferase
MES	2-morpholinoethanesulfonic acid
mRNA	messenger RNA
MS	Murashige and Skoog medium
OAA	oxaloacetate
ORF	open reading frame
P	phosphate
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PEPC	phosphoenolpyruvate carboxylase
PFP	pyrophosphate dependent phosphofructokinase
3-PGA	3-phosphoglycerate
Pi	inorganic phosphate
PPi	pyrophosphate
^{31}P-NMR	^{31}P nuclear magnetic resonance imaging
QPCR	quantitative PCR
RNA	ribonucleic acid
RSA	root system architecture
RT-PCR	reverse transcriptase PCR
RuBP	Ribulose biphosphate
SDS	sodium dodecyl sulphate
SSC	sodium aline, sodium citrate
S3P	shikimate 3-phosphate

ABSTRACT

Plant phosphate starvation responses are initiated which reduce Pi demand, conserve Pi reserves and increase Pi availability. Responses to Pi deprivation range from alterations in growth and metabolism to gene induction. Signalling pathways governing starvation responses are unknown, although individual responses have been found to be under the control of local phosphate availability, whilst other responses have been shown to be controlled by shoot-derived systemic signals. A comparison of the kinetics of different phosphate starvation responses, representing growth, physiology and gene expression, suggested that changes in gene expression and physiology occurred prior to growth responses. The magnitude of all responses was found to be dependent on the duration of starvation and on previous Pi growth conditions, suggesting that internal Pi levels regulate the initiation of responses. Kinetic studies investigating gene expression after Pi re-supply showed that down-regulation of expression is rapid and therefore possibly under local control. Enhanced Pi starvation responses were observed under increased sucrose availability, illustrating the importance of a balanced internal C:P ratio for plants. Split-root experiments revealed the systemic down-regulation of Pi-inducible genes and confirmed the existence of long-range signals governed by shoot Pi status in *Arabidopsis*. Experiments to determine the kinetics of down-regulation in split root plants showed that the extent of down-regulation was dependent on internal Pi status and on the duration of split-root treatment. Complete down-regulation occurred only after several days. Split-root experiments were conducted with various mutants to dissect signalling further. Wild-type down-regulation was observed in the phosphate response mutant, phr1-1, the cytokinin receptor mutant, cre1, and in *Arabidopsis* plants containing the Pi-inducible *At4* gene under the control of the CaMV 35S promoter. Reduced systemic down-regulation was observed in the phosphate translocation mutant, pho1, confirming that the signal for down-regulation is generated in response to shoot Pi levels. Down-regulation did not occur in another phosphate translocation mutant, pho2, despite high shoot Pi status. Pi sensing and Pi re-allocation are disrupted in this mutant, thus preventing the generation of a systemic down-regulation signal.

CHAPTER 1: INTRODUCTION

1.1 The importance of phosphate in plant nutrition

Phosphate is an essential plant macronutrient that comprises approximately 0.2% of total plant dry weight (Schachtman *et al.* 1998). It plays an important structural role in the formation of key biomolecules, connecting ribonucleoside units to form DNA and RNA macromolecules. Phosphate also forms a bridge between diglycerides and other molecules, such as alcohol, amino acids and amines, resulting in the formation of phospholipids, which are fundamental components of cellular membranes (Marschner, 1995). The role of phosphate is also critical in metabolic regulation and energy transfer in the form of phosphate, pyrophosphate, ATP, ADP and AMP. In addition, the regulation of various cellular functions requires post-translational protein phosphorylation-dephosphorylation by phosphate transfer (Ranjeva and Boudet, 1987). It is therefore evident that the availability of phosphate has major implications for plant growth and physiology.

1.2 Phosphate availability in soil

Although the total amount of phosphorus in soil may be high, it is often unavailable for uptake by plants. Plants can only take up phosphate in the inorganic form, orthophosphate (Pi). Most phosphate in soil (20 - 80%) is present in recalcitrant organic form, the main component of which is phytic acid, or inositol hexaphosphate (Schachtman *et al.*, 1998). Much of the remainder is adsorbed to surface-active sesquioxides and oxihydrates of clay minerals (Marschner, 1995). As a result, free inorganic phosphate rarely exceeds a concentration of 10 μM (Marschner, 1995). This is low considering the high phosphate requirements of plants, which must maintain a constant phosphate concentration of 5 – 20 mM within the cytoplasm (Bieleski, 1973). Soil inorganic phosphate is moved mainly by diffusion. However, plant roots take up Pi faster than can be supplied by slow diffusion rates of 10^{-12} to 10^{-15} m^2s^{-1}, resulting in a depletion zone around the root (Schachtman *et al.*, 1998; Lewis and Quirk, 1967). This can lead to phosphate levels at the root-soil interface further declining to submicromolar levels (Poirier and Bucher, 2002).

1.3 Phosphate in agriculture

Concentrated phosphate fertilisers are often applied to agricultural soils to ensure plant productivity. Raising the level of phosphate in soil improves the amount of phosphate available for uptake and also increases the rate of phosphate diffusion through soil (Bieleski, 1993). As much as 90 – 120 kg of phosphate fertiliser can be applied per hectare to intensely farmed soils. Unfortunately, fertilisers are often ineffective due to chemical immobilisation or further binding to soil particles and it is estimated that only 20% of applied phosphate gets absorbed by the annual crop growth (Vance, 2001). Environmental damage to lakes, rivers and marine estuaries can result from runoff of soil-applied phosphate. Eutrophication occurs as a result of excess phosphate concentration in water and is associated with algal blooms which deplete water oxygen levels, resulting in the death of many aquatic organisms (Killham, 1994). It is clear that other strategies for optimising phosphorous use need to be found and these may be developed through a greater understanding of how plants sense and respond to phosphate.

1.4 Phosphate transport from soil to cell

1.4.1 Transport of Pi into rhizodermal cells

Plants take up phosphate in the monovalent form, $H_2PO_4^-$. Uptake rates are greatest within a pH range of pH 5.0 and pH 6.0 at which $H_2PO_4^-$ is the dominant form of Pi. Plants acquire phosphate by an energy-mediated co-transport process with one or more protons (Ullrich-Eberius *et al.*, 1984). Energised transport from soil to plant is required to overcome the steep concentration gradient between high Pi present in plant tissues and low Pi available in the soil (Schachtman *et al.*, 1998). An electrochemical proton gradient generated by plasma membrane H^+-ATPases drives this process and absorption of Pi is accompanied by proton influx with a stoichiometry of 2 to 4 H^+ / $H_2PO_4^-$ (Ullrich-Eberius *et al.*, 1984; Sakano, 1990; Muchhal *et al.* 1996). Two types of uptake system exist, a low-affinity transport system that operates under high soil phosphate conditions (mM range) and a high-affinity transport system that operates at low phosphate concentrations (μM range). See section 1.6 for further details of phosphate transport and cellular ion homeostasis.

1.4.2 High-affinity phosphate transporters

The high-affinity transport system is considered to be the main mechanism for Pi acquisition at the root-soil interface, with Michaelis-Menten constants for high affinity Pi transporters ranging from 1.8 to 9.9 μM (Mimura, 1999). The structure of phosphate transporter proteins is highly conserved between many plant species and fungi and comprises twelve trans-membrane-spanning domains. A total of seventeen genes from several plant species have been identified as having a high degree of similarity to the yeast high-affinity phosphate transporter gene, *PHO84*. This group of genes has recently been classified as the *Pht1* family (Bucher *et al.*, 2001). Most members of the *Pht1* family of high-affinity phosphate transporters are expressed mainly in roots and are known to be inducible by Pi starvation. This includes *Pht1;1*, *Pht1;2*, *Pht1;3* and *Pht1;4* genes in *Arabidopsis thaliana* (Muchhal et al ., 1996; Smith *et al.*, 1997; Okumura *et al.*, 1998; Dong *et al.*, 1999, Mudge *et al.*, 2002;), *StPT1*, *StPT2* and *StPT3* in potato, *Solanum tuberosum* (Leggewie *et al.*, 1997; Rausch *et al.*, 2001), *MtPT1* and *MtPT2* in the model legume, *Medicago truncatula* (Liu *et al.*, 1998; Chiou *et al.*, 2001) and *LePT1* and *LePT2* in tomato, *Lycopersicon esculentum* (Liu *et al.*, 1997a; Daram *et al.*, 1998; Muchal and Ragothama, 1999). A phylogenetic tree of the nine *Arabidopsis Pht1* members can be seen in figure 1.1.

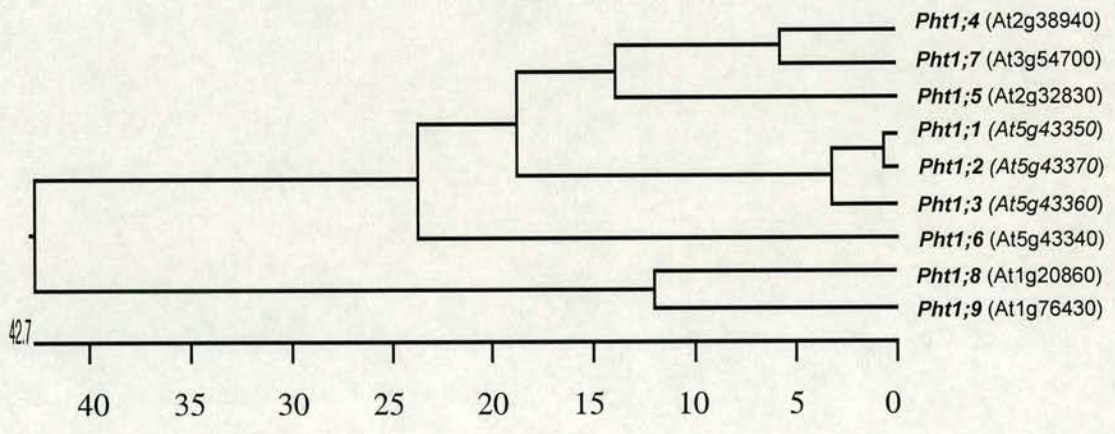


Figure 1.1: *Arabidopsis thaliana Pht1* phosphate transporter family phylogenetic tree, based on amino acid sequence alignment. TAIR accession numbers are shown in brackets.

Two of the *Arabidopsis Pht1* genes have been demonstrated to function as high-affinity phosphate transporters. *Pht1;1* and *Pht1;4* were able to complement the yeast *pho84* high-affinity Pi-transporter mutant (Muchhal *et al.*, 1996). In addition, *Pht1;1* over-expression was found to enhance Pi uptake into tobacco cells (Mitsukawa, *et al.*, 1997). GUS and GFP reporter genes driven by *Pht1* promoters have provided insights into the localisation of their expression during phosphate starvation. *Pht1;1* is expressed mainly in root hairs and emerging secondary roots, with strong expression in epidermal and endodermal layers. *Pht1;2* gene expression was observed in all cell types of the root meristematic region and in the epidermis, cortex and stellar region of the mature root (Karthikeyan *et al.*, 2002). Finally, *Pht1;3* expression was found in the stele and pericycle cell layer, whereas *Pht1;4* expression was observed in the stele, root tips and epidermis. Furthermore, the strongest *Pht1;1*, *Pht1;2* and *Pht1;3* expression was located in the root hair-forming trichoblast cells (Mudge *et al.*, 2002). This expression pattern has not been observed for other nutrient transporters and it emphasises the significance of root hairs in phosphate acquisition (see section 1.7.2).

Northern analysis of *Pht1;1* and *Pht1;2* mRNA levels demonstrated the phosphate-specific induction of these genes. Expression increased 5 to 8-fold after 3-4 days of Pi deficiency, compared to less than 2-fold under nitrogen and potassium deficiency (Smith *et al.*, 1997; Dong *et al.*, 1999). *Pht1;4* may be induced to even higher levels under Pi starvation. GUS and LUC activity was seen to increase as much as 100-fold after a similar length of Pi starvation in plants carrying the *Pht1;4* promoter fused to GUS and LUC reporter genes (Karthikeyan *et al.*, 2002). However, GUS and LUC proteins will be more stable than mRNA and will accumulate over time. Therefore, reporter gene activity may not accurately reflect the level of gene expression. This is supported by the observation that *Pht1;1* and *Pht1;4* mRNA expression, determined by Northern blot analysis, decreased substantially after one day of Pi re-supply, whereas luciferase activity in *Pht1::LUC* plants took several days to decrease to similar levels.

Dong *et al.*, 1999 found that increases in *Pht1;1* and *Pht1;2* expression were paralleled by increases in Pi uptake after a slight delay, presumably for synthesis of transporter protein. Once phosphate was re-supplied to Pi-starved plants, *Pht1;1* and *Pht1;2* expression decreased by 70% within a day. By contrast, Pi uptake remained at an elevated level for several days, suggesting a faster turnover rate for the mRNA transcripts compared to the protein.

1.4.3 Phosphate uptake via mychorrizal association

Many plants (> 80% of flowering plants) have the ability to obtain phosphate from distant soil areas via symbiotic associations with arbuscular mycorrhizal (AM) fungi, which colonise roots to utilise plant carbon resources. AM fungi take up phosphate from the soil and release it from the differentiated hyphae (arbuscules) that develop within the cortical cells of plant roots. A unique phosphate transporter, MtPT4, has been identified in *Medicago truncatula* that is only expressed in arbuscule-containing cells, specifically for the acquisition of Pi released by the fungus (Harrison *et al.,* 2002). *Arabidopsis* does not form mycorrhizal associations and much research into this form of Pi acquisition is conducted using a model legume, *M. truncatula.*

1.5 Phosphate translocation within the whole plant

1.5.1 Fate of phosphate after uptake into rhizodermal cells

According to Rausch and Bucher (2002) there are five possible destinations for Pi taken up from the soil into rhizodermal cells, it may: enter the cytoplasmic metabolic pool and be converted into organic compounds; become incorporated into phospholipids and nucleic acids via biochemical pathways; be transported into the vacuole for storage; or be lost via efflux out of the cell. Finally, in conditions of adequate phosphate, it may be transported symplastically to the xylem parenchyma cells and, from there, loaded into the xylem stream (as inorganic phosphate). It is therefore clear that, in addition to root-soil interface uptake systems, there are further transport systems that are involved in Pi translocation within the plant. These include phosphate transporters which participate in: the secretion of Pi into the root xylem stream, the loading of Pi from the xylem into leaf cells and loading from leaf cells into the phloem for Pi-cycling within the plant (Daram *et al.*, 1999).

1.5.2 Phosphate transport into the xylem stream

Xylem loading of phosphate in *Arabidopsis* is dependent, directly or indirectly, on *PHO1*, a gene that is expressed predominantly in root stelar cells (Hamburger *et al.*, 2002). Mutations in *PHO1* cause severely reduced Pi translocation to the shoot. The *pho1* mutant is formed by a single nuclear recessive mutation at the *PHO1* locus. The growth of this mutant is impaired and it displays visual phosphate starvation

characteristics, namely small leaves, thin stalks, delayed flowering, few secondary influorescences, an accumulation of anthocyanins in the leaves and stems, and the production of few, small seeds with reduced germination frequency. Absorption of phosphate by the root was found to be normal, whereas transfer to the shoots was reduced from 35% in the wild-type to only 0.9% in the *pho1* mutant (Poirier *et al.*, 1991). This finding, together with the localisation of *PHO1* in pericycle and xylem parenchyma cells, suggests a role for PHO1 in mediating the efflux of Pi from root stelar cells for loading into the xylem stream. *PHO1* is weakly up-regulated during Pi starvation and thus may play a role in mediating the faster rate of Pi transfer into the xylem stream that occurs under Pi stress. The PHO1 protein contains six potential membrane-spanning domains but shares no homology with any previously described transporter protein, instead sharing highest similarity with mouse and human retrovirus receptors. Therefore, *PHO1* may not itself be a transporter gene but may somehow indirectly influence the activity of a membrane Pi exporter (Hamburger *et al.*, 2002).

1.5.3 Phosphate transfer into leaves

Phosphate is transported from the xylem stream to younger leaves where it is mainly stored in vacuoles. It has been postulated that the *Pht2;1* low-affinity *Arabidopsis* phosphate-transporter plays a role in Pi transport into leaves because it has a high Km for Pi (0.4 mM) and is expressed strongly around leaf central vascular tissue. The *Pht2;1* protein is located in chloroplasts and is structurally similar to *Pht1* family members. It contains 12 trans-membrane (TM) domains but, unlike *Pht1*, it has a large hydrophilic region between the 8^{th} and 9^{th} TM domains. Despite possessing conserved amino acid regions found in yeast and mammal sodium-dependent Pi transporters, functional analysis of the *Pht2;1* protein in mutant yeast cells suggests that it is in fact a proton symporter (Daram *et al.*, 1999). *Pht2;1* is also distinct from *Pht*1 Pi- transporters by the observation that *Pht2;1* gene expression is not regulated by phosphate content. The *pht2;1* null mutant displays reduced growth and low leaf Pi content when grown under adequate Pi concentrations. This is similar to the effect of Pi limitation on photosynthesis, which is also thought to occur as a result of reduced Pi transport into chloroplasts (Versaw and Harrison, 2002).

Some members of the *Arabidopsis Pht1* high-affinity Pi-transporter family have little detectable expression in root tissue and are thus implicated in Pi transport within

the plant. RT-PCR expression analysis of Pi-deficient plants found very weak *Pht1;5* expression in roots but strong expression in cotyledons and old leaves. *Pht1;6* was also not expressed in roots even under extreme Pi starvation and was instead expressed in flower tissue. This indicates that these *Pht1* genes may actually function as low-affinity transporters involved in Pi loading of internal tissues (Mudge *et al.*, 2002).

1.5.4 Mobilisation and re-translocation of phosphate

Re-translocation of Pi from older leaves to growing shoots occurs under adequate phosphate conditions. There is also significant translocation of phosphate in the phloem from shoots to roots in the form of inorganic Pi (Jeschke *et al.,* 1997) and organic P compounds such as ATP and hexose-phosphates (Bieleski, 1969; Kluge *et al.*, 1970). Phosphate is transported into the phloem in the inorganic form but approximately half of that becomes incorporated into organic form whilst passing through the phloem (Bieleski, 1973). Under high Pi supply, much of the phosphate translocated to roots is recycled back to the shoots through xylem Pi transfer.

The supply of phosphate from roots to shoots is restricted under phosphate starvation and this is supplemented by mobilisation of phosphate reserves in older leaves followed by translocation to the younger leaves and growing roots (Schachtman *et al.* 1998). The sequential movement from the first to the second and then to the third leaves via movement through the apoplastic pathway from mesophyll cells to phloem, was visualised by ^{32}P pulse labelling of Pi-deficient barley plants (Mimura, 1995). Figure 1.2 illustrates the main flow of Pi between root and shoot cells.

Genes that may play a role in phosphate re-translocation include the *Pht2;1* *Arabidopsis* low-affinity Pi-transporter gene, as *pht2;1* mutants have reduced efficiency of Pi transfer from old to young leaves under phosphate starvation (Versaw and Harrison, 2002).

The *Arabidopsis* re-translocation mutant, *pho2*, is defective in the regulation of Pi accumulation in shoots. A mutation at the *pho2* locus causes seedlings to take up Pi at twice the wild-type rate and to accumulate a larger proportion of that in the shoots (2 to 5 fold that of wild-type). Re-distribution to roots is less than half that of wild-type plants (Delhaize and Randall, 1995; Dong *et al.*, 1998). Visible symptoms are dependent on transpiration rate, with leaves becoming necrotic under high transpiration conditions (Delhaize & Randall, 1995). Pi concentrations are equivalent in roots of wild-type and

pho2 mutants and the expression of *Pht1;1* or *Pht1;2* high-affinity Pi transporter genes remains unchanged in the mutant. Therefore, increased uptake by *pho2* mutants must be due to the enhanced expression of other Pi transporters or the allosteric activation of existing transporters (Dong *et al.*, 1998). The *PHO2* gene has yet to be cloned but the available evidence suggests an involvement in phloem transport possibly as a transporter of phosphate from shoot to root cells. Alternatively, PHO2 may play a role in phosphate sensing within cells, subsequently regulating the expression or activity of shoot Pi transporters (Dong *et al.*, 1998). If this is the case then PHO2 could be a possible regulator of *Pht*2;1 activity (Versaw and Harrison, 2002).

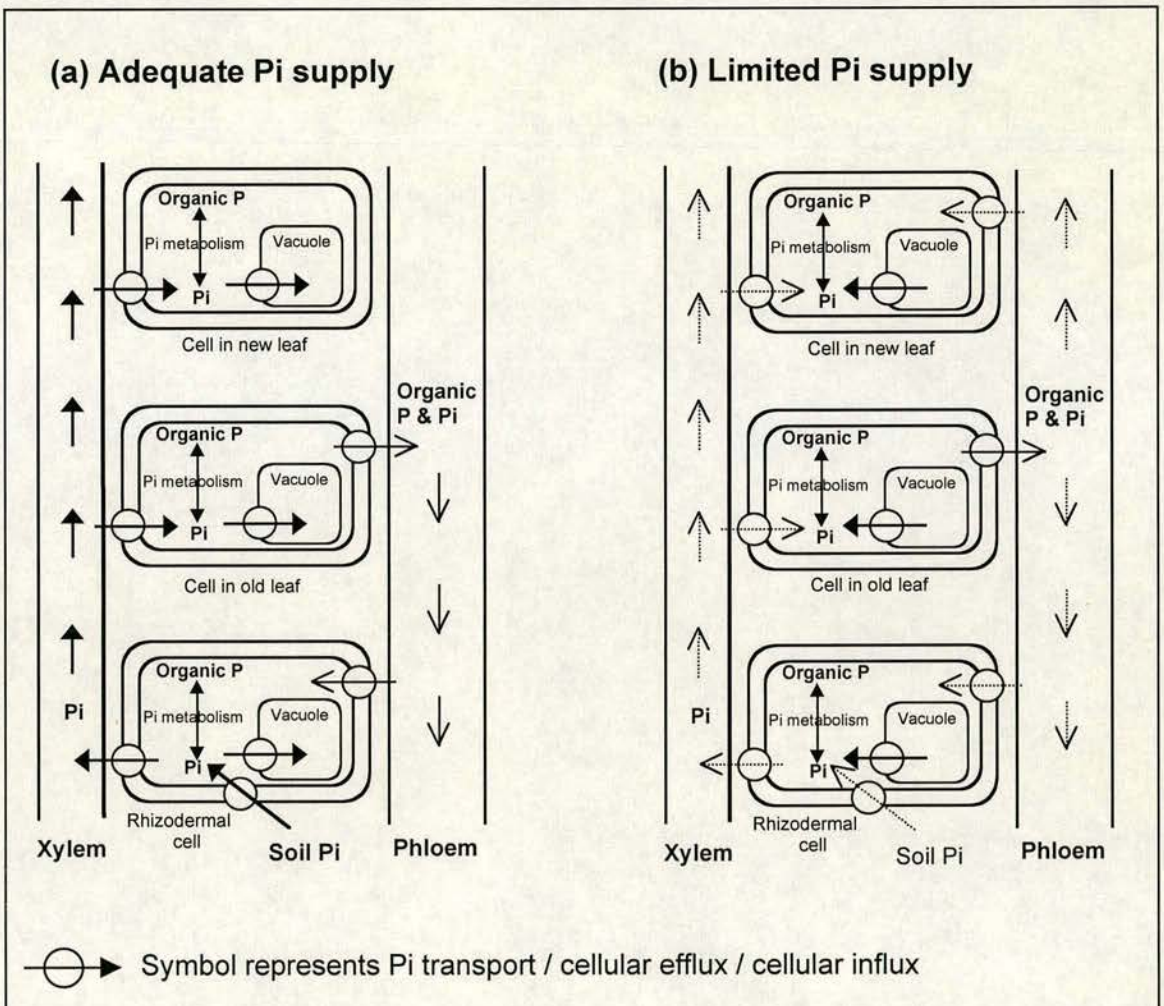


Figure 1.2: Schematic diagram representing the main phosphate translocation and re-translocation in plants under a) adequate Pi supply and b) limited Pi supply.

1.6 Phosphate transport and homeostasis at the cellular level

The concentration of inorganic ions in cellular compartments must be strictly regulated if they are to fulfill their roles effectively. Phosphate ion homeostasis is of particular importance because phosphate is present in inorganic and organic form and is therefore at a junction between inorganic and organic metabolism (Mimura, 1999). Phosphate homeostasis occurs via various cellular processes, such as chemical reactions with other inorganic ions, transport across membranes and metabolic conversions between inorganic and organic molecules. Phosphate is transported across the plasma membrane into the cytoplasm by H^+/Pi low- and high-affinity Pi transporters, depending on the location of the cell and the availability of Pi (see sections 1.4.2 and 1.5.3). There is some evidence that phosphate may also be co-transported across plasma membranes with Na^+. For example, *Chara corallina* internodal cells exhibit Na^+ - dependent Pi uptake. This has also been reported in yeast and in algae (Mimura, 1999). Some of the Pi transported into the cytoplasm will be metabolised and some will move back out of the cell for translocation to other plant tissues, although the molecular mechanisms mediating Pi efflux out of the cytoplasm are unknown. However, most will be transported across the tonoplast into the vacuole. The vacuole acts as a Pi reservoir and can contain approximately 85 - 95% of total plant Pi if it has adequate Pi supply (Bieleski and Ferguson, 1983). In contrast, cytoplasmic Pi levels remain constant and are not dependent on Pi supply. Under Pi deficiency, vacuolar levels of Pi will decrease to buffer cytoplasmic Pi levels (see figure 1.2). Phosphate levels in the cytoplasm will decrease only under extreme Pi stress once all vacuolar stores have been depleted. A vacuolar Pi transporter has yet to be identified but it is known that Pi transfer across the tonoplast requires ATP (Mimura, 1995).

Pi homeostasis is also achieved by regulated transport across membranes of other cell organelles. *Pht3* genes are involved in the transport of Pi across mitochondrial membranes. Inorganic Pi is transported across chloroplast membranes in exchange for photosynthetic intermediates. There are several classes of plastidic Pi transporters in *Arabidopsis* including: the phosphoenylpyruvate/Pi translocator encoded by the *PPT* gene; the triose-phosphate/Pi translocator encoded by the *TPT* gene; the glucose 6-phosphate/Pi translocator encoded by the *GPT* gene and the pentose phosphate /Pi translocator encoded by the *XPT* gene (Poirier and Bucher, 2002).

1.7 Morphological alterations in response to phosphate starvation

1.7.1 Changes in root system architecture

Plant growth is inhibited under phosphate-deficient conditions, with shoot growth being most affected. There is an increase in root-to-shoot dry weight ratio in phosphate-starved plants (Trull *et al.* 1997; López-Bucio *et al.*, 2002). Roots become the main sink for phosphate and photosynthates, resulting in increased root surface area which enhances Pi acquisition from soils (Marschner, 1995). The morphology of the root system changes dramatically under Pi deprivation. Lateral root growth is induced and lateral roots also increase in length and density, whereas cell expansion in the root apex becomes inhibited (Williamson *et al.*, 2001; López-Bucio *et al.*, 2002; Linkohr *et al.*, 2002). This results in greater root mass closer to the soil surface where phosphate is most abundant.

The mechanisms involved in the regulation of root system architecture (RSA) in response to phosphate are unknown. Regulation does not seem to occur through changes in photosynthate partitioning as the addition of sucrose does not change RSA (Williamson *et al.*, 2001). Neither does abscisic acid play a role (Trull *et al.*, 1997). The phytohormone, auxin, is necessary for lateral root development (Muday and Haworth, 1994; Reed *et al.*, 1998) and auxin-resistant mutants with reduced auxin sensitivity have fewer lateral roots (Estelle and Somerville, 1987). Despite previous observations that auxin application to plants reproduces Pi-deficient RSA, auxin has been ruled out as having any direct role because auxin-resistant mutants respond normally to phosphate concentration (Williamson *et al.*, 2001; Linkohr *et al.*, 2002). Recent research by López-Bucio *et al.* (2002) also confirmed this response and it was concluded that the corresponding genes were not directly involved in the lateral root growth response. However, they also discovered that low concentrations of applied auxin had the same effect in Pi-deficient seedlings that much higher concentrations did in Pi-sufficient plants, suggesting increased auxin sensitivity of plants grown under phosphate limitation. Therefore, auxin does have some role in phosphate-mediated RSA changes.

Another phytohormone, ethylene, may play a negative role in the formation of lateral roots as it inhibits their formation under high and low Pi conditions. However, ethylene-insensitive mutants have normal lateral root formation, suggesting that the involvement of ethylene in the lateral root response is indirect (López-Bucio *et al.*, 2002). Decreases in primary root length under Pi limitation are likely mediated by

ethylene action. Ethylene is necessary for cell expansion in primary roots under low Pi but is inhibitory under high Pi conditions. Thus, the availability of phosphate profoundly alters a plant's responsiveness to ethylene (Ma *et al.*, 2003).

Shoot phosphate status was suggested by Williamson *et al.* (2001) as having an important regulatory role in lateral root formation due to their finding that *pho2* shoot accumulation mutants had altered RSA. However, López-Bucio *et al.* (2002) discovered that *pho2* displayed wild-type RSA under low phosphate concentrations and argued that it was external, rather than internal, Pi levels that determined lateral root formation. It is also possible that lateral root elongation occurs as a secondary response to reductions in primary root growth rate. This would implicate the root tip as the main site for phosphate action with respect to RSA (Williamson *et al.*, 2001).

1.7.2 Root hair formation

Root hairs become more dense and elongated under low phosphate conditions, thus allowing exploration of a greater soil volume and an increase in the absorptive surface area of the root (Fohse *et al.*, 1991; Bates and Lynch, 1996). Root hairs were found to be 6 times longer in plants grown under low Pi compared to those in high Pi, achieved by an increase in both growth rate and growth duration (Bates and Lynch, 1996; Ma *et al.*, 2001). This increase in root hair length and density massively enhances root surface area from 0.21mm^2 mm^{-1} root under adequate Pi, to 1.44 mm^2 mm^{-1} root under Pi starvation. Autoradiography has shown that the Pi depletion zone around the root is influenced by root hair length (Lewis & Quirk, 1967; Bhat & Nye 1974, Misra *et al.*, 1988), proving that root hair growth under Pi starvation is an effective strategy for acquiring Pi from more distant soil areas. The high expression levels of the *Pht1* family of high-affinity H^+/symporters found in root hairs implies that this is the mechanism by which root hairs take up Pi from the soil (see section 1.4.2).

Epidermal cells located over the junction of two underlying cortical cells have the potential to become trichoblasts, or root-hair forming cells. Epidermal cells form root hairs if the CTR1 (constitutive triple response) gene becomes inactivated through exposure to an ethylene precursor (ACC) at the anticlinal walls of cortical cells (Tanimoto *et al.*, 1995; Dolan, 1996, Schiefelbein, 2000). Root cross-sectional analysis demonstrated the existence of extra cortical cells in plants grown under low Pi conditions (Ma *et al.*, 2001). This leads to an increased number of epidermal cells

positioned over cortical cell junctions, thereby increasing the number of cells that can convert to trichoblasts. However, not all cells in this location will form root hairs. Up to 90% form root hairs under phosphate starvation but only 24% do under adequate phosphate conditions. It is therefore thought to be a combination of more cells being located over cortical cell junctions, plus an increased number of these cells forming root hairs, that contribute to greater root hair density under phosphate limitation. Cortical cell number remains stable in known root hair mutants and under ethylene and auxin treatments. Therefore, increased cortical cell number is likely brought about by a specific effect of low Pi on meristem activity (Ma *et al.*, 2001).

It has been proposed that ethylene may mediate adaptive morphological responses to Pi stress such as the formation of root hairs (Lynch and Brown, 1997). Ethylene precursors (ACC) applied to roots result in further root hair cells developing in the positions normally occupied by atrichoblasts, the non root hair-forming cells. In addition, ethylene application to plants promotes root hair elongation (Tanimoto *et al.*, 1995; Dolan, 1996; Pitts *et al.*, 1998). However, the application of an ethylene synthesis inhibitor (AOA) had no effect on root hair density in low phosphate seedlings. If ethylene is important in mediating the root hair response to phosphate then reduced root hair density should have been observed under this treatment. Therefore, a different signalling pathway may be involved (Ma *et al.*, 2001).

Auxin also plays a role in root hair formation. IAA (auxin) exposure promotes root hair elongation elongation under high Pi conditions (Pitts *et al.*, 1998). Also, auxin transport inhibitors dramatically reduce root hair elongation under low Pi (Bates and Lynch, 1996). Yet, wild-type root hair elongation was induced by low Pi in auxin insensitive and auxin resistant mutants. This suggests that the mechanisms responsible for increased root hair density in high Pi plants after auxin treatment are different to those involved in the low Pi response.

1.8 Molecular responses to phosphate starvation

Many genes that are induced or up-regulated in phosphate-limited plants have recently been identified. Some of these genes have been well characterised but the functions of others remain unknown. In addition, the functions of some phosphate starvation-induced molecules have been well studied but the genes involved are still unknown.

1.8.1 Phosphate transporter genes

The *Pht1* gene family encodes several high-affinity transporters, many of which are up-regulated under phosphate limitation to enhance Pi uptake into rhizodermal cells. See section 1.4.2 for further information.

1.8.2 Extracellular and intracellular phosphate mobilisation and liberation

Many plants respond to phosphate starvation by increasing the production of extracellular compounds that enhance phosphate mobilisation in the soil (Goldstein *et al.*, 1998; Haran *et al.*, 2000; Baldwin *et al.*, 2001; Miller *et al.*, 2001). Recently, acid phosphatase secretion from *Arabidopsis* roots was visualised in plants transformed with a construct containing the promoter of a cloned acid phosphatase gene fused to GFP (Haran *et al.*, 2000). Root secretion of acid phosphatases is thought to play a role in hydrolysing and mobilising Pi from organic phosphates present in the soil, or to salvage Pi from phosphate esters which have been leaked by plant cells (Duff *et al.*, 1994). One gene known to code for acid phosphatase in *Arabidopsis* is the *AtACP5* gene, a member of the purple acid phosphatase family. Members of this family have been discovered in a wide range of animal and plant species. Northern analysis found enhanced *AtACP5* expression in both root and aerial tissues after 2 days of phosphate starvation, with further increases in expression up to 10 days after transfer to Pi deprivation. *AtACP5* is also induced to some extent under salt, abscisic acid and peroxide stresses. The observation that *AtACP5* is expressed in roots, and is likely localised to the cell wall or plasma membrane, suggests a possible role in phosphate scavenging from the soil. However, the further observation of expression in shoot tissue hints at a broader role. It has been suggested that AtACP5 may additionally be involved in phosphate scavenging within plant tissues (del Pozo *et al.* 1999). Intracellular acid phosphatases are ubiquitous within all plant tissues and are hypothesised to play a role in recycling of phosphate and mobilisation of Pi from phosphate-containing cellular compounds. Subcellular fractionation studies have found intracellular acid phosphatases to be mainly confined to the vacuole, although some are located in the cytoplasm. The precise role of acid phosphatases in the vacuole is somewhat unclear considering that vacuolar phosphate stores are mainly in inorganic form. Possibly they are involved in the sequestration of phosphate from compounds that may be toxic to cells (Duff *et al.* 1994).

Another *Arabidopsis* acid phosphatase gene, *PUP1*, has been identified as having a putative role in soil phosphate mobilisation. *PUP1* is induced by phosphate starvation, while plants carrying mutations in this gene are defective in the production of secreted acid phosphatase. The *phosphatase-underproducer* (*pup1*) mutant was discovered by its inability to cleave phosphorous from BCIP (5-bromo-4-chloro-3-indolyl-phosphate), a reaction that normally turns the indicator compound blue. The *pup1* mutant has a normal phenotype with the exception of a slight reduction in the root-to-shoot ratio during phosphate limitation (Trull and Deikman, 1988). The inactivation of one acid phosphatase enzyme is therefore not enough to substantially inhibit growth, suggesting the existence of multiple Pi-regulated phosphatase enzymes.

The *Arabidopsis pho3* mutant was also identified using BCIP as an indicator of secreted phosphatase activity. Unlike the *pup1* mutant though, *pho3* has a distinct phosphate starvation phenotype including reduced growth, increased anthocyanin accumulation and defective Pi uptake. This indicates that PHO3 may have a role in phosphate signalling or in the regulation of internal phosphate levels. Observations of decreased phosphatase activity are probably only secondary effects (Zakhleniiuk *et al.*, 2001).

Nucleic acid-degrading enzymes are also induced during phosphate starvation. Chen *et al.* (2000) used this as a basis to screen for *Arabidopsis* phosphate starvation response mutants. They identified 22 mutants that were unable to utilise nucleic acids as a sole phosphate source. One example of this is the *psr1* (*phosphate starvation response 1*) mutant that is defective in both acid phosphatase and ribonuclease enzyme activities. This mutant carries a single recessive mutation in the PSR1 gene. Therefore, PSR1 likely affects the expression of multiple genes coding for nucleic acid-degrading enzymes normally induced during phosphate starvation.

The *Arabidopsis* phosphate starvation-inducible *RNS1* gene specifically codes for a ribonuclease that is involved in the liberation of phosphate from RNA to facilitate its re-mobilisation. *RNS1* is highly induced in wild-type plants after 7 days of phosphate starvation. Expression levels are also high in the constitutively phosphate-starved *pho1* mutant. Based on its protein structure, RNS1 is likely to be extracellular. Thus, RNS1 may be responsible for the degradation of RNA from lysed cells or for RNA degradation in the soil, if secreted from the root. Another ribonuclease-encoding gene, *RNS2*, is also induced by phosphate deprivation. RNS2 is thought to be localised to the vacuole and

responsible for RNA degradation in that cellular compartment. The *RNS* genes are also somewhat upregulated during senescence, presumably to re-mobilise phosphate from macromolecules in dying cells (Bariola *et al.,* 1994).

Another method plants use to liberate phosphate from Pi-containing compounds is by increasing apyrase activity. Apyrase enzymes are involved in the hydrolysis of phosphate from extracellular ATP and ADP. Over-expression of a *Pisum sativum* apyrase was found to enhance greatly phosphate accumulation and subsequent growth in ATP-fed transgenic plants. Apyrase may therefore function to capture phosphate from ATP released by soil flora (Thomas *et al.*, 1999).

Organic acid production is also increased under phosphate limitation. Citrate, malate and oxalate are exuded from roots to enhance the availability of phosphate in the rhizosphere by displacing phosphate from insoluble inorganic complexes (Lipton *et al.*, 1987; Hoffland *et al.*, 1989). Organic acid biosynthesis occurs during non-photosynthetic carbon fixation via phospho*enol*pyruvate carboxylase (PEPC). It is increased PEPC activity that accounts for more organic acid production under Pi starvation (Johnson *et al.*, 1996a, 1996b; Neumann and Romheld., 1999; Aono *et al.*, 2001). Citrate exudation, in particular, greatly increases in phosphate-limiting circumstances. It was found to be present in the exudates of plants at significantly greater concentrations in Pi -deficient *Medicago sativa* seedlings (Lipton *et al.*, 1987). Citrate and malate are both exuded by *Arabidopsis* roots but the relative amounts differ significantly depending on the *Arabidopsis* accession studied (Narang *et al.*, 2000). Transgenic tobacco plants, carrying the *Pseudomonas aeruginosa* citrate synthase gene under the control of the 35S CaMV promoter, had 2 to 4 fold enhancement of citrate excretion. They also accumulated 15% more shoot biomass and up to 35% more fruit dry weight than control plants (López-Bucio *et al.*, 2000). Contradictory results were recorded by Delhaize *et al.* (2001). The same technique was used to create tobacco plants that over-produced citrate by 100-fold but no increase in citrate accumulation or efflux was observed. This may be due to differences in experimental environments affecting the expression of the transgene, or it is possible that the increased biomass recorded in the original experiment was due to some other unknown effect such as the location of the transgene in the genome.

1.8.3 Alterations in membrane composition

Photosynthetic membranes are rich in non-phosphorous glycolipids. These include the sulfolipid, sulfoquinovosyldiaglycerol, and the galactolipids, mono- and digalactosyldiaglyserol. Phospholipids such as phosphatidylglycerol are also present but these become a source of phosphate under Pi deprivation conditions. The ratio of non-phosphorous to phosphorous-containing lipids increases under phosphate starvation as sulfolipids and galactolipids replace membrane phospholipids.

The *Arabidopsis SQD1* gene encodes a protein involved in the first stage of sulfolipid biosynthesis. Northern blot analysis showed increasing *SQD1* mRNA expression with decreasing Pi concentration (Essigmann *et al.,* 1998). *SQD2*, a gene required for the second step in sulfolipid biosynthesis, is also induced under phosphate limitation. Mutations in this gene result in plants completely devoid of sulfolipid. The *sqd2* mutants grow normally in adequate Pi conditions. However, growth is impaired under Pi starvation conditions because phosphate reserves in the form of membrane phospholipids cannot be replaced or subsequently utilised. This indicates that sulfolipids are conditionally important, acting as a surrogate for thylakoid membrane phospholipids when phosphate becomes limiting (Yu *et al.*, 2002).

MGDG synthases, responsible for monogalactosyldiaglycerol (MGDG) synthesis, exist as two types, type A (encoded by *MGD1*) and type B (encoded by *MGD2* and *MGD3*). Type A enzyme is most abundant and is expressed at high levels in leaf chloroplast membranes but remains unchanged by phosphate availability. On the other hand, expression of *MGD2* and *MGD3* is stimulated by Pi starvation. Transcripts of *MGD2* and *MGD3* are mainly detected in non-photosynthetic tissues, with *MGD2* mostly in inflouresences and *MGD3* mostly in roots. Their predicted location is in the outer membrane of the plastid envelope (Awai, *et al.*, 2001). MGDG is necessary for digalactosyldiaglycerol (DGDG) synthesis. Enhanced MGDG production under phosphate deprivation will therefore result in increased levels of (DGDG). This step requires the action of DGDG synthase (DGD1). Mutations in the *DGD1* gene led to a 90% reduction in DGDG content. Unexpectedly, this effect was rescued in the *dgd1* / *pho1* double mutant. DGDG fatty acid composition in the double mutant was found to be different to that of original DGDG and was indicative of ER-derived DGDG rather than plastid-derived DGDG. Therefore, phosphate deprivation is thought to redirect galactolipid synthesis from the plastid pathway to the ER pathway and increased ER-

derived DGDG substitutes for extraplastidic membrane phospholipids such as phosphatidylcholine (Härtel *et al.*, 2000; Klaus *et al.*, 2002).

1.8.4 Genes with unknown function

Several genes with unknown function are also induced during phosphate starvation. These include four genes belonging to a small gene family, *AtIPS1* and *At4* (or *AtIPS2*) in *Arabidopsis* (Burleigh and Harrison, 1997; Martin *et al.*, 2000), *Mt4* in *Medicago truncatula* (Burleigh and Harrison, 1997) and *TPSI1* in tomato (Liu *et al.*, 1997). Expression of these genes increases specifically under phosphate limitation. They are characterised by their structure, each consisting of several, short overlapping open reading frames (ORFs). These ORFs are not conserved between members of the gene family, with the exception of a single 4 amino acid peptide, MAIP, which is found in both AtIPS1 and At4. These two *Arabidopsis* genes also share some similarity at the nucleotide level, with one stretch of 57 nucleotides being completely identical. A region of 22 nucleotides within this stretch is highly conserved (95% identity) between other members of the family. Root-specific *Mt4* and *At4* expression was observed by Burleigh and Harrison (1997), whereas Martin *et al.* (2000) found *At4* expression in both roots and shoots, similar to *AtIPS1* and *TPSI1* expression. The function of this gene family remains unknown although it has been suggested that the encoded peptides play a role in mediating the phosphate starvation response. However, given the scarcity of homology between peptides in this family, it is possible that they do not function at the protein level. Instead, the RNA molecules themselves may be the active component. Analysis of known *Arabidopsis* EST sequences has identified non-coding RNAs (ncRNAs) as an emerging class of transcripts. Fifteen putative ncRNAs have been identified in existing literature (the *At4/ AtIPS1/ Mt4/ TPSI1* family included) and many more have been identified using computational analysis tools. Many ncRNAs are thought to play important roles in a wide rage of cellular processes and most were found to be plant-specific (MacIntosh *et al.*, 2001).

Another gene with unknown function that is induced by phosphate starvation is the *Arabidopsis PSR3.2* gene, which encodes a β-glucosidase. Beta-glucosidases belong to the BGA (β-glycosidase A) family that also encompasses the galactosidases and the phosphoglucosidases. BGA family members are differentially regulated under changing environmental conditions. *PSR3.2* was shown to be strongly specific to Pi-starvation,

with most expression in roots. PSR3.2 may perform a metabolic or regulatory role. Evidence for a metabolic role includes the observation that expression levels increase in response to phosphate deprivation prior to any alteration in plant growth. Also, PSR3.2 has strong similarity to other β-glucosidases that are involved in metabolism and regulation. However, it is also highly homologous to a *Brassica napus* β-glucosidase that has an inactive cytokinin as a substrate and thus instead may be involved in growth regulation (Malboobi and Lefebvre, 1997).

The addition of Pi to tobacco BY2 cell cultures results in the transient expression of *PHI1* and *PHI2* genes. Their functions are unknown but they are induced by ABA treatment and share homology with transcription factors involved in ABA-signalling pathways (Sano *et al.*, 1999; Sano and Nagata, 2002)

1.9 Metabolic alterations in response to phosphate limitation

Phosphate is a crucial component for many aspects of plant metabolism (see section 1.1). Phosphate limitation can result in major alterations to photosynthesis, carbon fixation, glycolysis and respiration.

1. 9.1 The effect of phosphate limitation on photosynthesis and carbon fixation

Photosynthesis has been found to be limited by Pi supply (Sivak and Walker, 1986). Photosynthetic carbon assimilation (the 'source') is regulated by the demand for carbon assimilates (the 'sink'). Low sink strength under phosphate starvation is thought to be responsible for photosynthetic limitation whereby a decrease in sucrose synthesis, due to low plant demand, restricts Pi recycling to the chloroplast. This in turn limits RuBP regeneration and ATP synthesis and thus reduces the rate of photosynthesis (Pieters *et al.*, 2001). Additionally, phosphate deprivation can affect the thylakoid energy-transducing systems. It can alter the structural assembly of photosynthetic apparatus and it can also inhibit key enzymes in the Calvin cycle (Poirier and Bucher, 2002, Hurry *et al.*, 2000, Härtel *et al.*, 1998). Reductions in photosynthetic electron transport have also been reported in the constitutively phosphate-starved *Arabidopsis* mutant, *pho1* (Hurry *et al.* 2000).

Photosynthesis is reliant on membrane lipids. Alterations in membrane lipid composition, such as phospholipid replacement with sulfolipids and galactolipids, allow photosynthesis to proceed under phosphate stress (see section 1.8.3).

1.9.2 The effect of phosphate limitation on glycolysis

Pi levels in the cytoplasm can decrease under extreme phosphate stress. This will occur if vacuolar Pi reserves are exhausted and can no longer buffer cytoplasmic levels. Decreases in cytoplasmic Pi have profound effects on glycolysis because many enzymes involved in this process require inorganic Pi, ATP or ADP as co-substrates. Several enzymes are activated during phosphate deprivation to substitute for adenylate or inorganic Pi-requiring enzymes (see figure 1.3). *Arabidopsis* UDP-glucose phosphorylase is induced under Pi starvation and substitutes for ATP-requiring hexose kinase, utilising pyrophosphate (PPi) as an alternative substrate (Ciereszko *et al.* 2001). In *Brassica nigra* cell suspension cultures, PFP, non-phosphorylating NADP-G3PDH and PEP-phosphatase can catalyse alternative reactions to bypass PFK, phosphorlyating NAD-G3PDH and 3-PGA-kinase, or PK, respectively. Activity of bypass enzymes was found to increase by at least 10-fold under phosphate starvation (Duff *et al.*, 1989; Moraes and Plaxton, 2000). Conversely, the activity of the adenylate and inorganic Pi-requiring enzymes remained uniform during this time, presumably to ensure that normal respiration would quickly be restored were more phosphate to become available (Duff *et al.* 1989). It has also been suggested that PFP, PEPC and PEP phosphatase may function to recycle Pi from esterified phosphate to produce inorganic Pi which can then be assimilated back into metabolic pathways (Theodoru and Plaxton, 1993). Interestingly, PEPC is also involved in the production of organic acids that are excreted into the environment enhancing phosphate availability (see section 1.8.2).

1.9.3 The effect of phosphate limitation on respiration

Mitochondrial respiration is also affected by phosphate starvation. Electron flux through the cytochrome pathway is reduced when levels of ADP and Pi become limiting. The existence of two alternative non-phosphorylating electron transport pathways contributes to the survival of plants during Pi deprivation because they circumvent the ADP and Pi-requiring steps (Fig. 1.3). These are the cyanide-resistant alternative oxidase pathway and the rotenone-insensitive bypass to complex I (Theodoru and Plaxton, 1993).

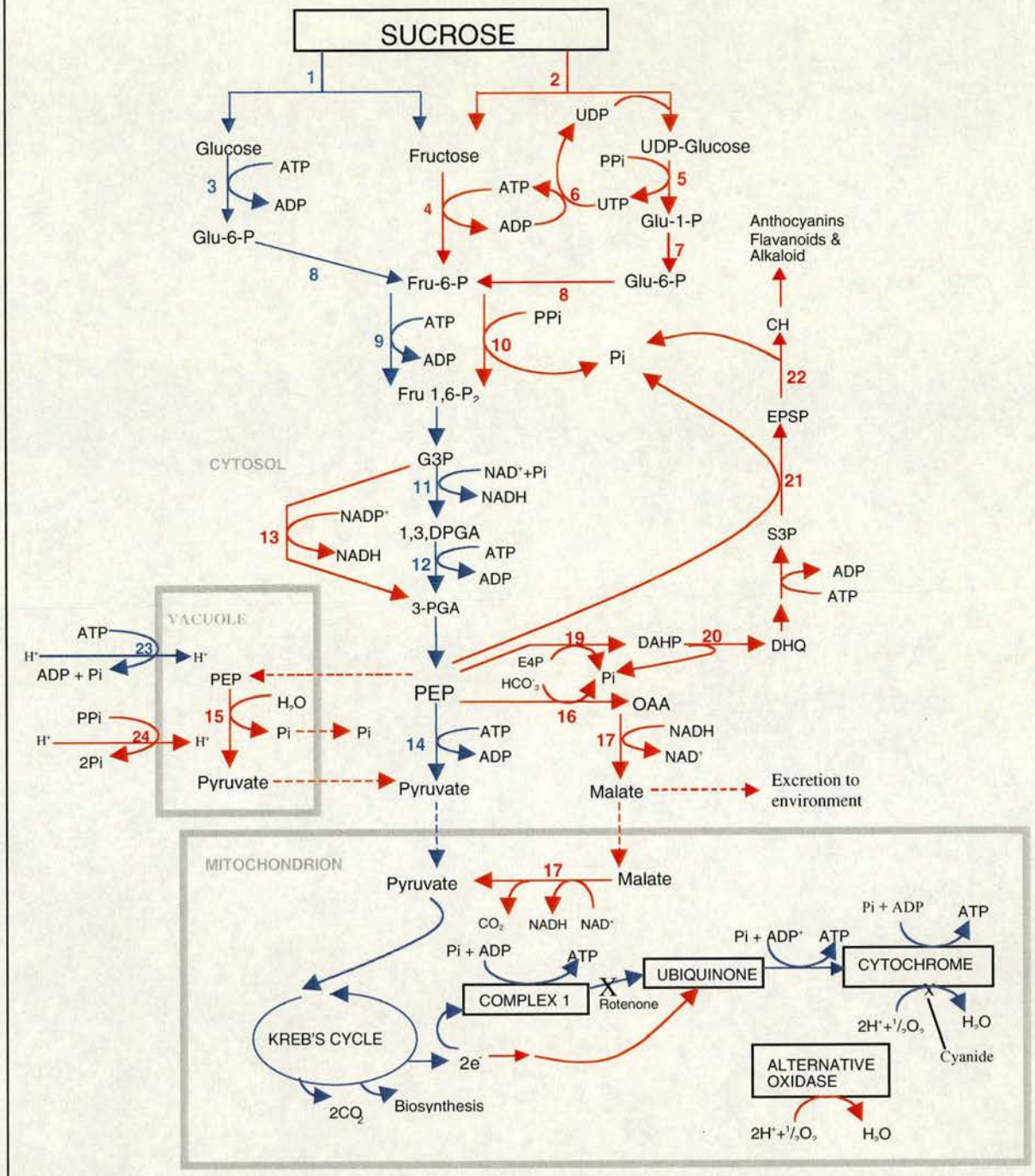


Figure 1.3: Alternative metabolic pathways during phosphate starvation. Red arrows indicate modified pathways. The enzymes that catalyze the numbered reactions are: 1, invertase; 2, sucrose synthase; 3, hexokinase; 4, fructokinase; 5, UDP-glucose pyrophosphorylase; 6, nucleoside diphosphate kinase; 7, phosphoglucomutase; 8, phosphoglucose isomerase; 9, ATP-dependent phosphofructokinase; 10, pyrophosphate-dependent phosphofructokinase; 11, NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (phosphorylating); 12, 3-phosphoglycerate kinase; 13, NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (non-phosphorylating); 14, pyruvate kinase; 15, phosphoenolpyruvate phosphatase; 16, phosphoenolpyruvate carboxylase; 17, malate dehydrogenase; 18, malic enzyme; 19, 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthase; 20, 3-dehydroquinate dehydratase; 21, 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase; 22,chorismate synthase; 23, tonoplast H+-ATPase; 24, tonoplast H+-pyrophosphate. 1999). Adapted from Plaxton and Carswell (1999)

1.9.4 Anthocyanin accumulation during phosphate limitation

Anthocyanin production is enhanced in plants experiencing phosphate stress, resulting in the development of dark green or purple leaf colouration. Susceptibility to nucleic acid damage and photinhibitory damage to chloroplasts may occur during Pi limitation. Anthocyanins absorb UV light and are thus expected to protect against such damage (Theodoru and Plaxton, 1993).

1.10 Signalling during phosphate starvation

Plants have evolved a multitude of responses to cope with phosphate starvation, from alterations in growth and metabolism to the induction of many genes. Some of these responses may be secondary effects of another phosphate starvation response. For example, changes in metabolism are linked to changes in gene expression. Likewise, growth is associated with metabolic rate and may also be affected by the induction of certain genes. It is clear that a complex signalling network must exist to co-ordinate the various levels of the phosphate starvation response. However, despite extensive characterisation of plant responses to phosphate stress, the signals involved remain unknown.

1.10.1 The yeast Pho regulon

Yeast and bacteria have evolved similar adaptive responses to phosphate starvation, including the induction of high-affinity phosphate transporters and secretion of phosphatases (Vogel and Hinnen, 1990; Hulett, 1996; Torriani-Gorini, 1994; Lau *et al.*, 2000). The regulatory networks involved in these responses are well studied. The yeast Pi-starvation response is of particular interest due to the possibility that some signalling components may be conserved in other eukaryotic organisms such as plants. The Pho regulon of the yeast, *Saccharomyces cerevisiae,* is composed of 22 genes. The signal or receptor for Pi has yet to be identified but the signal transduction pathway involved in regulating the yeast phosphate starvation response is well characterised (Fig. 1.4). Two basic mechanisms control the response, phosphorylation/ de-phosphorylation and the compartmentation of a transcription factor. Under high Pi conditions, the Pho80/ Pho85 cyclin-CDK complex phosphorylates the Pho4 transcription factor on five

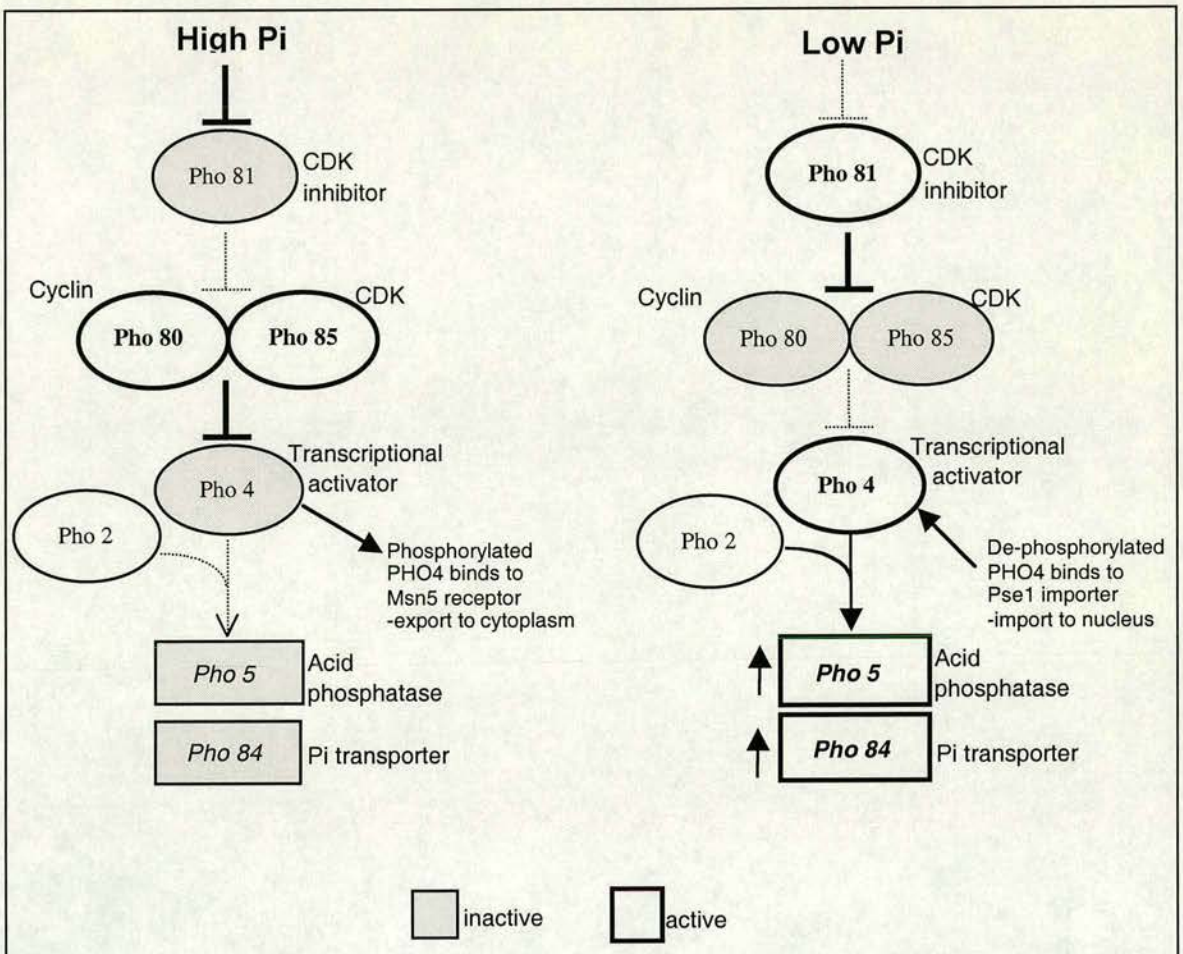


Figure 1.4: The *Sacchoromyces cerevisiae* Pho regulon. Ovals represent proteins and rectangles represent genes. Thick lines represent signals transduced downstream. Dotted lines represent absence of downstream interaction. Diagram adapted from Ogawa et al. (2000)

serine residues (Lenburg and O'Shea, 1996; Komeili and O'Shea, 1999). Phosphorylated Pho4 binds to a shuttling receptor, Msn5, which exports Pho4 from the nucleus to the cytoplasm (Kaffman et al., 1998a). Exclusion from the nucleus prevents Pho4 from interacting with Pho2, resulting in the termination of the phosphate starvation response. During phosphate limitation, the Pho81 CDK inhibitor down-regulates the kinase activity of the Pho80/ Pho85 complex, preventing Pho4 phosphorylation (Oshima *et al.* 1996). De-phosphorylated Pho4 binds to an import protein, Pse1, and is re-imported into the nucleus where it associates with Pho2, a second transcription factor (Kaffman *et al.*

1998b). The Pho2/ Pho4 complex binds with the promoters of *Pho5* and *Pho84* to activate their transcription (Hirst *et al.*, 1994). *Pho5* encodes an acid phosphatase and *Pho84* encodes a high-affinity phosphate transporter (Lenburg and O'Shea, 1996; Oshima *et al.*, 1996). It is possible that a phosphate regulon such as that found in yeast may also operate in plants. The induction of acid phosphatases and high-affinity phosphate transporters under phosphate deprivation are responses that are conserved between the two organisms. In fact, genes that encode high-affinity Pi transporters in plants were originally isolated due to their homology to the yeast *Pho84* gene. However, signalling and regulation of responses will ultimately be more complex in multicellular organisms because different organs, tissues and cells will experience varying Pi levels at any one time. Different signalling pathways may therefore operate at the local and systemic level.

1.10.2 A MYB transcription factor involved in plant phosphate-starvation signalling

The *Chlamydomonas rheinhardtii PSR1* gene was the first gene identified as a Pi-starvation signal in photosynthetic eukaryotes. Mutations in this unicellular algal gene caused a reduced rate of Pi transport and lower acid phosphatase production. The PSR1 protein was found to contain MYB DNA-binding domains. Expression of *PSR1* increased 13-fold after 8 hours of Pi starvation (Wykoff *et al.*, 1999). The *Arabidopsis phr1* mutant, isolated during a screen for Pi-response mutants, was found to possess a mutation in a gene homologous to *PSR1*. Activity of all Pi-starvation response genes tested was found to be reduced in this mutant. The PHR1 protein also contains a MYB domain and a predicted coiled-coil domain for protein interaction. PHR1 binds as a dimer to an imperfect palindromic sequence found to be present in all tested phosphate starvation-response genes. This indicates that PHR1 acts downstream in the Pi-starvation signalling pathway and may be a component of the plant phosphate regulon. Expression of *PHR1* remained relatively unchanged under different Pi conditions and the Pi content of the *phr1* mutant was reduced even under high Pi. PHR1 may therefore be involved in the regulation of Pi status at all times, regardless of plant Pi content. Alternatively, post-translational modification of PHR1 may occur or it may interact with another component of the signalling pathway (Rubio et al, 2001).

C. rheinhardtii PSR1 and *A. thaliana PHR1* genes may be potential components of a plant phosphate regulon. If this is so, then a very different form of regulation occurs

in plants compared to that of the yeast PHO regulon. Firstly, PSR1 and PHR1 share no similarity with any yeast protein. Also, both proteins are permanently resident in the nucleus (Wykoff *et al.*, 1999; Rubio *et al.*, 2001). In contrast, nuclear-cytoplasmic shuttling of the yeast PHO4 transcriptional activator is central to the regulation of the yeast phosphate starvation response. Finally, Mukatira *et al.* (2001) discovered that regions of the promoter of two Pi-starvation response genes, *Pht1;4* and *TPS11*, specifically bound nuclear protein factors from Pi-sufficient plants but not from Pi-starved plants. This implies that genes induced by Pi starvation are under negative control, whereas activation of yeast Pi-starvation response genes is under the control of the positive regulator, PHO4 (Rausch and Bucher, 2002). Therefore, the proposed plant phosphate regulon is likely to differ somewhat from the yeast PHO regulon.

1.10.3 Local signalling

Different cells and tissues of the plant may encounter varying phosphate availability. The existence of local or cell-autonomous signalling pathways, activated in response to immediate Pi conditions, would allow localised responses to be initiated faster than those controlled by global or whole-plant signalling events. Evidence for the existence of a separate local signalling pathway was provided by root hair studies. *Arabidopsis* roots grown on high Pi media were found to have short root hairs. Small sections of the same root that were exposed to low Pi concentrations grew significantly longer root hairs, thus proving that root hair elongation is initiated in response to local Pi availability. However, the signals leading to the response are unknown. It is also unclear whether local signalling events are cell autonomous or not. In this experiment the entire root tip was in contact with low Pi, therefore the Pi sensing mechanism could be located anywhere within the root tip, on the outside of cells, or inside the cells themselves (Bates and Lynch, 1996).

The fact that root hair elongation is a local response appears logical. Soil is extremely heterogeneous and neighbouring parts of the root system may experience very different Pi availabilities. Increasing root hair length in a poor Pi micro-environment will allow greater exploration of the soil and will also enhance the surface area of that root hair for Pi uptake.

1.10.4 Systemic signalling

Phosphate starvation signalling at the level of the whole plant would require a mobile systemic signalling molecule. Split root studies provide evidence for systemic signalling during phosphate starvation. Burleigh and Harrison (1999) split the root system of a *Medicago truncatula* plant into two containers, one half containing high Pi and the other low Pi. The expression of *Mt4*, a phosphate starvation-inducible gene of unknown function, was down-regulated in the starved half of the root system. The expression of *At4*, an *Arabidopsis Mt4*-like gene, was also analysed. The application of Pi-fertiliser resulted in reduced *At4* expression in wild-type plants. This did not occur in the *Arabidopsis pho1* translocation mutant, suggesting that the signal for down-regulation is dependent on translocation of Pi to the shoot. Split root experiments conducted with tomato plants reached similar conclusions. Expression of the high-affinity phosphate transporters, *LePT1* and *LePT2*, was down-regulated in the Pi-starved half of the root system when the other half was exposed to adequate Pi concentrations (Liu *et al.*, 1998b). It is therefore apparent that some responses to phosphate availability are regulated by systemic signals generated depending on internal shoot Pi status.

1.10.5 Putative phosphate starvation signals: Phosphate?

Phosphate is a highly mobile nutrient that is transported throughout the plant. Translocation rates are altered during phosphate limitation, thus raising the possibility that phosphate itself may be the systemic signal. To test this theory, phosphate levels were measured in each half of the root system during split root experiments conducted with *M. truncatula*. The side of the root system receiving high Pi fertiliser accumulated high amounts of Pi, whereas the side of the root system receiving low Pi fertiliser did not. It was concluded from this experiment that phosphate could not be the signal for down-regulation of Pi-starvation gene expression in *M. truncatula* plants (Burleigh and Harrison, 1999).

1.10.6 Putative phosphate starvation signals: The *At4/ AtIPS1/ Mt4/ TPSI1* family?

The function of the *At4/ AtIPS1 /Mt4 / TPSI1* gene family is as yet unknown. One potential function of this family may be as phosphate-starvation signalling molecules. Each gene comprises several short, non-conserved, overlapping ORFs (see section 1.8.4). This capacity to encode small peptides, together with the fact that up-regulation is

specific to phosphate stress, make them good candidates as downstream signalling molecules (Burleigh and Harrison, 1999). Indeed, another gene with similar structure, *ENOD40*, encodes short peptide signals involved in growth regulation of nodule development (van de Sande *et al.*, 1996, Compaan *et al.* 2001, Rohrig *et al.*, 2002).

More recently it has been proposed that the RNA molecules themselves, rather than the peptides, are the active component because there is a lack homology between members of the *At4/ AtIPS1/ Mt4/ TPSI1* gene family at the protein level (Poirier and Bucher, 2002). Database analysis has identified many more non-coding RNAs (ncRNAs). Of those ncRNAs that have been characterised, some are involved in gene regulation and are components of RNA-protein complexes that perform a variety of enzymatic and structural roles (MacIntosh *et al.* 2001). Therefore, the structure of short, overlapping ORFs found in *At4/ AtIPS1/ Mt4/ TPS11* family is not necessarily indicative of a signalling role, although it cannot be ruled out as a possibility.

It is also possible that this family of genes encode miRNAs (micro RNAs) which are involved in post-transcriptional gene silencing (PTGS) in a manner similar to siRNA (short-interfering RNA). miRNAs are composed of 19-25 nucleotides and their formation requires cleavage of stem-loop RNA by a Dicer homologue (Dicer is an element also required for siRNA formation). miRNAs then target complementary sequences for cleavage, thereby mediating gene-silencing. However, unlike siRNA, miRNAs are single-stranded molecules. The role of miRNAs in plant developmental processes is currently the focus of intensive research, although no direct role has so far been demonstrated (Hunter and Poethig, 2003). It remains to be seen whether they may also play a role in responses to environmental stress.

1.10.7 Putative phosphate starvation signals: Cytokinin?

Application of exogenous cytokinins to Pi-deprived *Arabidopsis* plants resulted in the repression of the Pi-starvation response genes, *At4*, *AtIPS1*, *AtACP5* and *Pht1;1*. Conversely, root hair number and length, a response controlled by local phosphate concentration, remained unchanged by cytokinin application. This implicates cytokinin as a potential systemic signal governing molecular responses to the Pi status of the whole plant (Martin *et al.* 2000). This may be of biological relevance considering that cytokinin is known to reduce root growth under nutrient stress. Also, levels of

endogenous cytokinin decrease in plants grown under phosphate-starvation conditions (El-D, 1979; Kuiper *et al.*, 1988)).

Recently, CRE1 was identified as a cytokinin receptor. It is activated by the perception of cytokinins to initiate phosphorelay signalling (Inoue *et al.* 2001). Expression of *CRE1* was shown to be downregulated in response to phosphate limitation and induced by cytokinins. Repression of Pi-starvation response genes by cytokinin is reduced in plants carrying mutations in *CRE1*, thus confirming that cytokinin plays a role in the negative control of phosphate-starvation gene expression (Franco-Zorrila *et al.* 2002).

Cytokinins are important phytohormones that participate in a variety of plant processes including seed germination, chloroplast differentiation, organogenesis and cell cycle control. In addition, the cytokinin: auxin ratio is critical in the development of roots and shoots and their relative growth (Haberer and Kieber, 2002). There is also some speculation that cytokinin could be a root-shoot signal involved in communicating nitrogen availability (Sakakibara *et al.*, 2000; Takei *et al.*, 2002). It seems likely that if cytokinins are involved in signalling phosphate starvation, they may serve as a more general nutrient stress response. Cytokinin signal transduction occurs via two-component circuitry, whereby a histidine kinase receptor (such as CRE1) initiates a phosphorelay that enables response regulators to act as transcriptional regulators. Response regulators belonging to the *ARR* gene family have been shown to be up-regulated by cytokinins (D'Agostino *et al.* 2000; Hwang and Sheen, 2001) and also by nitrate (Taniguchi, 1998). One member of the *ARR* family, *ARR6*, was recently found to be up-regulated by phosphate, nitrate and potassium deprivation (Coello and Polacco, 1999). This again points to cytokinin as possibly having a more general nutrient response role.

1.10.8 Putative phosphate starvation signals: Auxin/ Ethylene?

Auxin application to phosphate-starved plants has no effect on the expression of Pi-starvation response genes (Martin *et al.* 2000). Therefore, auxin does not appear to have a role in mediating gene expression in response to Pi status of the plant. However, auxin, along with ethylene, does play a role in root hair elongation and lateral root hair formation. These phytohormones are likely to be involved in signalling pathways leading to alterations in root system architecture. However, understanding of how they

mediate RSA changes in response to phosphate limitation is fragmentary (Abel *et al.*, 2002). Further details can be found in section 1.7.

1.11 Cross-talk with other nutrient signalling pathways?

Maintaining a balance of nutrients is necessary for survival. The nutritional status of a plant is dependent on the co-ordination of different adaptive responses to each nutrient, the availability of which will vary to different degrees.

The root system architecture (RSA) of *Arabidopsis* plants differs with nitrate and phosphate availability. Increases in Pi concentration result in longer primary roots and fewer lateral roots, whereas increases in nitrate concentration lead to shorter primary roots with no alteration in lateral root formation (Linkohr *et al.*, 2002). The signalling pathways governing these responses are unknown but it seems likely that there must be some form of cross-talk mechanism that co-ordinates the root response to both nutrients.

Carbon availability appears to have no effect on root/ shoot resource allocation during phosphate limitation (Paul and Stitt, 1993; Williamson *et al.*, 2001). However, high sucrose: nitrogen ratios were recently found to repress lateral root initiation. Thus, a signal transduction pathway that integrates information about both nutrients is responsible for mediating lateral root formation (Malamy and Ryan, 2001). In addition, increases in sucrose: nitrogen ratios have been shown to enhance nitrate-starvation responses such as anthocyanin accumulation. Further reductions in photosynthetic gene expression and chlorophyll content were also observed (Martin *et al.*, 2002). The ratio of these two nutrients is therefore important in regulating both growth and gene expression.

The carbon: phosphate ratio also has implications for gene expression. Several sugar-inducible genes, including the soybean vegetative storage proteins, *VspA* and *VspB*, were found to be transcriptionally activated by phosphate depletion. This discovery was attributed to changes in metabolism brought about by the effect of sucrose on cellular phosphate pools (Sadka *et al.*, 1994). More evidence of converging signal transduction pathways in response to carbon availability and other stresses was provided by Ho *et al.* (2001) who found that a random selection of environmental stress-related genes were negatively regulated by sucrose concentration.

The expression of several genes with a potential role in mineral nutrition signal transduction was studied in tomato under different phosphorous, iron and potassium nutritional regimes. These included MAP kinases, a putative transcription factor and a 14-3-3 protein. Many were up-regulated within a few hours in response to all three nutritional stresses, thus providing further evidence of cross-talk between different nutritional signalling pathways. These genes may well be general stress response genes but it is also possible that they are elements common to different signalling systems (Wang *et al.*, 2002). The combination of two nutrient-limiting conditions creating a synergistic effect on gene expression has also been documented by Huang *et al.* (2000), who discovered that zinc deficiency up-regulates the expression of high affinity phosphate transporters in barley plants.

Current knowledge regarding signalling pathways participating in the regulation of responses to any one particular nutrient is incomplete. More evidence of cross-talk between different nutrient signalling pathways is also emerging but the mechanisms involved remain equally unclear.

1.12 Objectives

The aim of my research was to investigate further plant responses to phosphate starvation and to dissect signalling pathways involved in the initiation of these responses. Previously documented plant responses to phosphate availability often focus on one particular response, be it growth, metabolism or gene expression. I aimed to characterise the phosphate starvation response in detail by observing the kinetics of various phosphate starvation responses in the model plant, *Arabidopsis thaliana*. The kinetics of primary root growth, root cortical cell size and the induction of several Pi starvation response genes were observed and compared. The order and timing of initiation of different responses shed further light on plant responses to short and long term Pi starvation. Another aim of my research was to determine the effect of carbon availability on phosphate starvation responses to further investigate potential cross-talk between the carbon and phosphate signalling pathways. The main emphasis of my research was to dissect phosphate starvation signalling. Systemic signals have previously been found to down-regulate the expression of Pi starvation response genes in other species. Therefore, a split-root experiment was designed to determine the existence of

systemic signalling in *Arabidopsis* plants. Quantitative RT-PCR analysis of gene expression in different halves of a root system receiving different Pi treatments uncovered the existence of systemic control of Pi starvation response genes. Finally, a selection of mutants, including the phosphate translocation mutants, *pho1* and *pho2*, the cytokinin receptor mutant, *cre1*, the phosphate signalling mutant, *phr1-1*, and a line transformed with the *At4* gene under the control of the CaMV 35S promoter were also subjected to split-root treatment. The presence, absence or alteration of systemic down-regulation in these mutants provided further insights regarding the nature of the systemic signal.

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant growth methods

2.1.1 Seed surface sterilisation

Arabidopsis seeds were surface sterilised by shaking in bleach solution (conc. NaOCl diluted 1 in 5 with sterile H_20, plus 20 µl Triton-x 100) for 10 minutes followed by several washes with sterile H_20. Seeds were re-suspended in 0.1% agar (micro agar, Duchefa) and stratified at 4°C for 2 days.

2.1.2 Plant growth media

All plants were grown in half-strength Murashige and Skoog (1962) media, prepared from the following components (chemicals supplied by Fluka): 100x micro-elements (10 mM H_3BO_3, 19.2 nM $CoCl_2$, 9.34 nM $CuSO_4.5H_2O$, 11.08 mM $Na_2EDTA.2H_20$, 9.99 mM $FeSO_4.7H_20$, 100 mM $MnSO_4$. H_20, 0.1 mM $NaMoO_4.2H_20$, 0.5 mM NaI, 4.8 mM $ZnS0_4.7H_20$); 10x macro-elements (29.9 mM $CaCl_2$, 15 mM $MgSO_4$, 206 mM NH_4NO_3, 188 mM KNO_3, 12.5 mM KH_2PO_4); 50x sucrose (30% sucrose), 50x MES (0.5 M MES, titrated with NaOH to pH 5.5). Phosphate concentration was altered by replacement of 10x macro-elements (complete) with 10x macro-elements (minus KH_2PO_4). Low KH_2PO_4 media was supplemented with KCl to maintain a constant K^+ concentration. Media were titrated to pH 5.6 using 0.1 M KOH. Media were solidified with 1% w/v micro agar (Duchefa).

2.1.3 Phosphate assay

The Murphy and Riley (1962) method of phosphate determination was adapted to assess the phosphate contribution by 1% micro-agar to plant growth media. The Pi content of molten micro-agar (1% w/v in H_20) was compared to a series of KH_2PO_4 standards (0 – 50 µM KH_2PO_4 in H_20). Samples and standards (10.5 ml each) were incubated in screw-capped tubes at 55°C and 2 ml of freshly prepared Reagent B (105.6 mg L-ascorbic acid dissolved in 20 ml Reagent A) was added. Reagent A was prepared by adding 6 g NH_4 molybdate tetrahydrate and 145.4 mg potassium antimony tartrate to 500ml 5 N H_2SO_4, followed by dilution with deionised water to 1 litre. Samples were incubated at 55°C for 1 hour. Absorbance was measured at a wavelength of 698 nm. The mean A_{698} of molten

1% micro-agar was found to be 0.116, equivalent to 7.6 μM KH_2PO_4. Therefore, agar-solidified media contain 7.6 μM Pi more than the stated phosphate concentration.

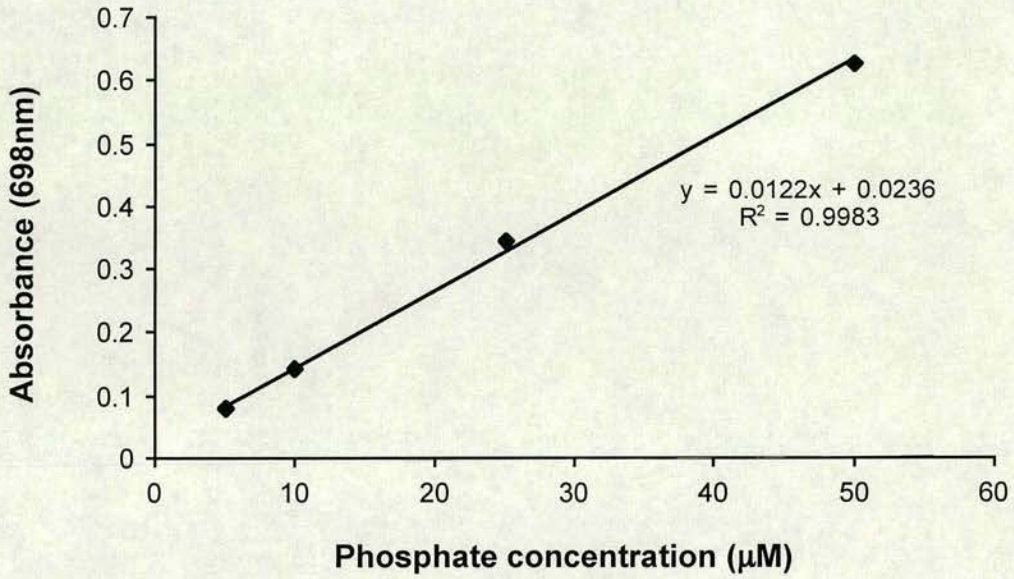


Figure 2.1: Absorbance of KH_2PO_4 standards for phosphate assay. Standards were prepared in H_20 and incubated for 1 h with Murphy and Riley Reagent.

2.1.4 Root growth assays

Seedlings were germinated and grown on square plates containing 75 ml agar-solidified 0.5x MS media (no Pi, 125 μM Pi or 2.5 mM Pi at 0.3%, 2% and 3% sucrose w/v). Seeds were sown in a row 2 cm from the top of the plate. Plates were sealed with parafilm and placed in a vertical position to encourage root growth along the surface of the media. Growth conditions were a 16 h day / 8 h night photoperiod, at 21°C. The position of the primary root tip was marked after 5 days and then every subsequent 24 hours. Measurements from approximately 30 seedlings in each growth condition were pooled.

Similar growth conditions were applied to determine root growth responses after transfer to starvation conditions. Seedlings were germinated and grown as above in 62.5 μM Pi or 125 μM Pi (0.6% sucrose w/v). Root tip position was marked every 12 h from day 5 to day 7. On day 7, seedlings were transferred to fresh vertical plates containing

no Pi or the original Pi concentration (0.6% sucrose w/v). Before transfer, roots were rinsed in liquid media containing the same Pi concentration as the destination plates. Root tip position was marked on transfer and every 12 h after transfer.

2.1.5 Measurement of root cortical cell length

A transgenic *Arabidopsis* line containing a GFP::PIP2A fusion protein was used for cortical cell size measurements. Line Q8 (Columbia background) expresses a GFP fusion to the plasma membrane water channel protein PIP2A (Cutler *et al*, 2000) thus allowing clear visualisation of root cell size (figure 2.2a). Roots were visualised under x 20 magnification using a confocal microscope equipped with UV filter (Olympus Inverted System Microscope IX70). Q8 root growth assays were performed to confirm that growth responses were similar to wild-type (figure 2.2b). For cortical cell measurements, Q8 seedlings were grown under the same Pi and sucrose regimes as for wild-type root growth assays (see section 2.1.4).

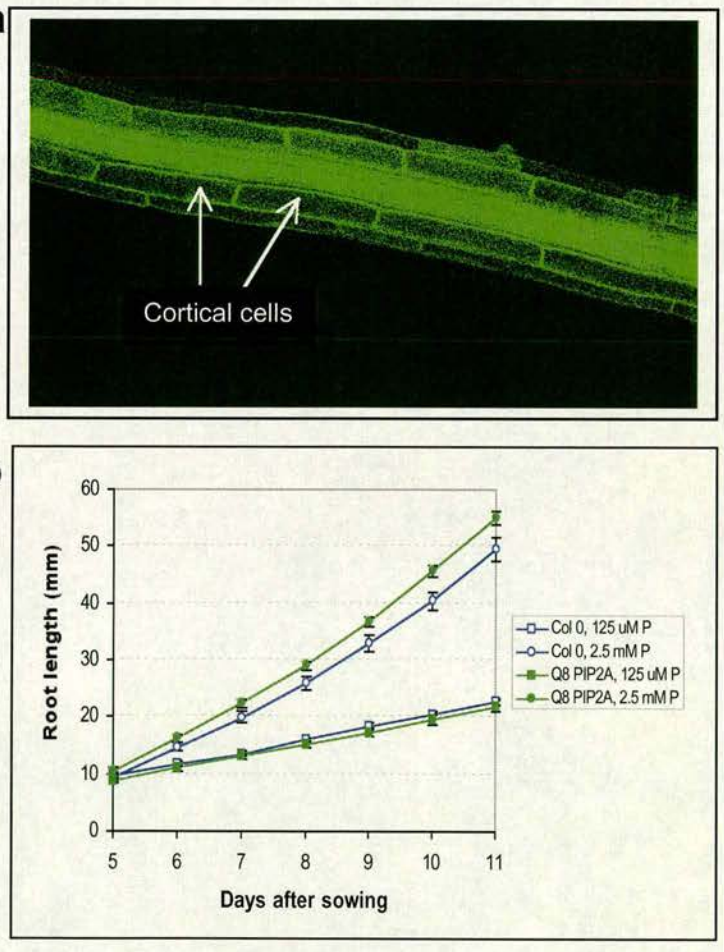


Figure 2.2: *Arabidopsis* GFP Q8 PIP2A seedlings

a) confocal image of GFP Q8 PIP2A roots visualised with UV filter, showing cortical cell location.

b) Primary root length of GFP Q8 PIP2A seedlings compared to wild-type seedlings. Seedlings were grown in vertical plates containing media of varying Pi concentration. Data represents mean of 25—3(seedlings +/- 1 SE.

eliminate the chance of including elongating cells. Flouview image analysis software was used to calculate cortical cell length.

2.1.6 Plant growth conditions for molecular characterisation experiments

For molecular characterisation of steady state gene expression, root tissue was harvested from seedlings grown under root growth assay Pi and sucrose conditions (see section 2.1.4), 5, 7 and 11 days after sowing.

For kinetic analysis of gene expression after transfer to Pi starvation, 10-15 seedlings were germinated and grown in conical flasks containing 5 ml 0.5 x MS liquid media containing 125 μM or 2.5 mM Pi (0.3% sucrose w/v). Flasks were stoppered with a foam bung to maintain sterile culture conditions whilst allowing gas exchange between the flask and the external environment and placed on a shaker (40 rpm) at 21°C with a 16 h day / 8 h night photoperiod. Liquid media were replenished 6, 8 and 10 days after sowing. On day 11, liquid media were replaced with no Pi media (0.3% sucrose w/v). Roots were rinsed with 5 ml 0.2 mM $CaCl_2$ (in 5 mM MES, p H5.5) to complex residual phosphate ions. Roots were rinsed once more with no Pi media and then replaced with fresh no Pi media. Root tissue was harvested from one original flask prior to media replacement (t=0) and at various time intervals after media replacement.

For kinetic analysis of gene expression after Pi re-supply, seedlings were transferred to no Pi liquid media, as above, and grown for 5 days. Media were then replaced with 2.5 mM Pi media (0.3% sucrose w/v). Root tissue was harvested from one flask prior to media replacement (t=0) and at various time intervals after media replacement. Root tissue was frozen in liquid nitrogen at harvest for RNA extraction.

2.1.7 Split-root experiments

Seedlings were germinated and pre-grown in vertical plates containing 75 ml 125 μM Pi agar-solidified media. All media used in split-root experiments contained 0.3% sucrose. After 6 days, primary roots were cut off just below the hypocotyl to stimulate growth of lateral roots. Seedlings were grown for a further 7 days, then transferred to vertical plates containing fresh 125 μM Pi media. After 7 days, seedlings were transferred to 3-compartment petri-dishes, one seedling per plate. Shoots were placed on the surface of one compartment containing 18 ml agar-solidified no Pi media. The root system was

separated equally into the two remaining compartments filled with 12 ml liquid media. For steady state analysis, one half of the root system was divided into either 125 μM Pi or 2.5 mM Pi media and the other half of the root system was placed in no Pi media. Controls included seedlings with roots divided into no Pi, 125 μM Pi or 2.5 mM Pi media in both halves. Plates were sealed with parafilm and placed on a shaker (40 rpm) at 21°C with a 16 h day / 8 h night photoperiod. Root and shoot tissue was harvested separately after 5 days. For kinetic analysis of gene expression in split-root plants, seedlings were grown with half their root system separated into 2.5 mM Pi and the other half of the root system in no Pi media. Control seedlings had both halves of the root system divided into no Pi or 2.5 mM Pi media. Root tissue was harvested after 3, 6 and 9 days. Liquid media were replenished every 3 days. Tissue samples were frozen in liquid nitrogen at harvest for RNA extraction.

Phosphate assays were performed on liquid media at time of sampling to ensure against Pi contamination (0.5x MS liquid media, containing a series of Pi concentrations, were used as standards, see section 2.1.3 for method.).

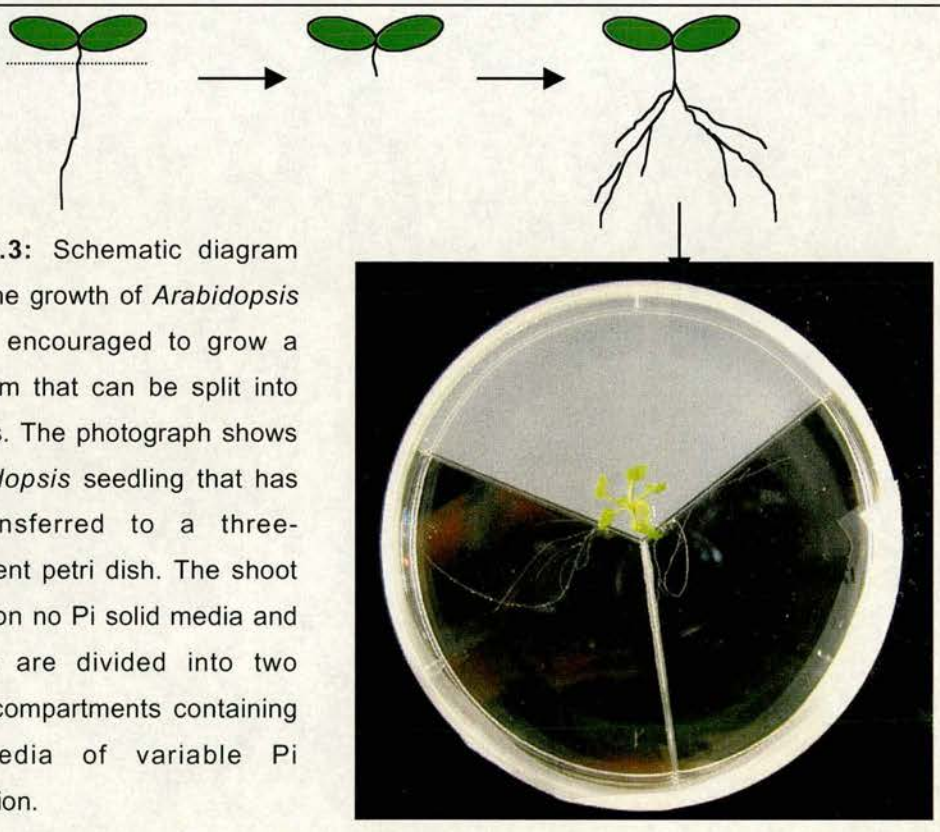


Figure 2.3: Schematic diagram showing the growth of *Arabidopsis* seedlings encouraged to grow a root system that can be split into two halves. The photograph shows an *Arabidopsis* seedling that has been transferred to a three-compartment petri dish. The shoot is resting on no Pi solid media and the roots are divided into two separate compartments containing liquid media of variable Pi concentration.

2.1.8 Growth and analysis of *At4* promoter::GFP plants

Arabidopsis seedlings homozygous for the *At4* promoter::GFP construct were germinated and grown on vertical plates containing 2.5 mM Pi media for 10 days and then transferred to vertical plates containing no Pi media. Roots were visualised using a confocal microscope equipped with UV filter (Olympus Inverted System Microscope IX70) 24 h after transfer and then every subsequent 24 h.

2.2 RNA Methods

2.2.1 RNA isolation

The method of total RNA isolation was as follows for a 100 mg sample. The method was scaled up or down according to sample weight. Tissue was homogenised in TRIzol reagent (95 ml H_20-saturated phenol (38%), 23.63 g guanidine thiocyanate (0.8 M), 7.61 g ammonium thiocyante (0.4 M), 8.4 ml 3 M sodium acetate, pH 5 (0.1 M), 12.5 ml glycerol, plus H_20 t0 250 ml). After addition of 200 µl chloroform, samples were vortexed (15 s), left at room temperature for 2-3 minutes, then centrifuged at 14000 rpm for 15 minutes at 4°C. The aqueous phase was removed. To precipitate RNA from the aqueous phase, 0.8 M Na citrate/ 1.2 M NaCl (half the volume of the aqueous phase) was added, followed by the same volume of isopropanol, then left at room temperature for 10 minutes. Samples were then centrifuged at 14000 rpm (Eppendorf centrifuge 5417C) for 10 minutes, at 4°C. RNA pellets were washed with 1 ml 75% ethanol in DEPC-H_20, briefly vortexed, then centrifuged again at 14000 rpm (Eppendorf centrifuge 5417C) for 10 minutes at 4°C. RNA pellets were briefly air-dried then re-suspended in 50 µl DEPC-H_20. RNA was re-precipitated with 2 M LiCl (final volume) overnight at 4°C, followed by centrifugation at 14000 rpm (Eppendorf centrifuge 5417C) for 30 minutes at 4°C. RNA pellets were air-dried then re-suspended in 50 µl DEPC-H_20.

2.2.2 Quantitative RT-PCR analysis

RNA was reverse-transcribed using Invitrogen Life Technologies Superscript™ First-Strand Synthesis System for RT-PCR, using oligo (dT) primers. Approximately half a microgram of RNA was reverse-transcribed per half RT reaction. cDNA was diluted to 80 µl with sterile H_20 and 3 µl of diluted cDNA were used per 20 µl Quantitative PCR (QPCR) reaction. QPCR reaction components included 3 µl cDNA sample, 10 µl 2x Abgene QPCR Master Mix (containing 0.025 units/µl Thermo-Start® Enzyme), 4.2 µl sterile H_20, 2 µl SYBR® Green 1 (1:10000 dilution of 10000x concentrate in DMSO, Molecular Probes), 0.4 µl (100 ng/µl) forward primer and 0.4 µl (100 ng/µl) reverse primer (see table 2.1). Each QPCR reaction was prepared in quadruplicate and then aliquoted into 96 well PCR plates, which were sealed with optical sealing tape. QPCR reactions were amplified using the iCycler iQ™ Real-Time Detection System (BIO-

RAD), using the 490 nm filter for SYBR green fluorescence detection. PCR reactions consisted of an initial 15 min. denaturation step (which was also necessary to activate the Thermo-Start® Enzyme) followed by 40 cycles of melting at 95°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s.

Each cDNA sample was amplified with *eIF 4A* (cDNA) primers to assess total RNA content. The *eIF 4A* gene encodes the constitutively expressed eukaryotic protein synthesis initiation factor 4A (Metz *et al.*, 1992). Amplification with *eIF 4A* (genomic DNA) primers was also performed to assess genomic DNA contamination when carrying out PCR with *At4* primers (*At4* mRNA is intron-less). All other primer combinations contained at least one primer designed to cross an intron border and thus only cDNA should have been amplified.

cDNA	Primer sequence for Quantitative PCR	
eIF 4A (cDNA)	Forward	5' TTCGCTCTTCTCTTTGCTCTC 3'
	Reverse	5' GAACTCATCTTGTCCCTCAAGTA 3'
eIF 4A (genomic DNA)	Forward	5' CATTTTCTCCGCACATCATC 3'
	Reverse	5' AAACTGTGTGCCTTCTGGTG 3'
At4	Forward	5' GGATGGCCCCAAACACAAG 3'
	Reverse	5' TAAACCGGAAACAAAGTAAACACG 3'
AtIPS1	Forward	5' TTGGGCAACTTCTATCCTTTGG 3'
	Reverse	5' GCAAATTTACATGCACTGGTCTG 3'
MGD3	Forward	5' TGCCACCGTACATGGTTC 3'
	Reverse	5' TTGTCCTATTGGATTACTTTCTTTAGAG 3'
SQD1	Forward	5' CTTAGCAAAGTTCATGATTCGCAC 3'
	Reverse	5' GCCTCTCGTCTGACCACCTTTAC 3'
Pht1;1	Forward	5' CTGCCAAGCTGATTAAGAGC 3'
	Reverse	5' GACAGAGCACAAGATCATCATTAC 3'
AtACP5	Forward	5' CACGGCGAGTCTGAGTTTGCTG 3'
	Reverse	5' CTCTCCAATTTTTCCCATCTGATAAGC 3'
PEPc	Forward	5' GAGTATTTCCGCCTCGCTAC 3'
	Reverse	5' CTTGGCGAACACCATTTC 3'

Table 2.1: Primer sequences for amplification of cDNA by Quantitative PCR

The threshold value was kept constant for all samples. To normalise for cDNA loading, the mean threshold cycle of amplification for each gene was subtracted from the mean threshold cycle of *eIF 4A* amplification from the same cDNA sample (this value was known as the 'threshold cycle difference'). One sample per experiment was chosen as the base level to which all other samples were compared. This sample was always the sample with the largest calculated threshold cycle difference (i.e. the sample with the lowest gene expression). Therefore, the threshold cycle difference for each sample was subtracted from the largest threshold cycle difference value. The remaining value was the exponent of 2 (as each cycle involves the doubling of cDNA amounts of the previous cycle). This is simplified in the equation below:

Fold difference in gene expression = $2^{c-(b-a)}$

a = mean threshold cycle of amplification for tested gene
b = mean threshold cycle of amplification for *eIF 4A* gene (cDNA)
c = largest threshold cycle difference value

2.2.3 Northern Blot Analysis

Separation of 15 µg RNA was performed in a 1% formaldehyde agarose gel (Sambrook *et al.* 1989). The gel and running buffer contained 1x MOPS (Sambrook *et al.* 1989). After electrophoresis, gels were washed with DEPC-H_20 for 3 x 15 min., then 10x SSC in DEPC-H_20 (Sambrook *et al.* 1989) for 2 x 15 min, with slow shaking. RNA was blotted onto a nylon membrane overnight, then UV cross-linked and immobilised at 90°C for 1 h. The nylon membrane was hybridised to a ^{32}P-labelled 358 bp *At4* cDNA fragment as described by Sambrook *et al.* 1989. RNA loading was determined by hybridisation to the constitutively expressed eukaryotic translation initiation factor, *eIF4A*. Blots were hybridised at 65°C and washed once at room temperature with 2x SSC, 1% SDS, and once at 65°C with 2x SSC, 1% SDS, followed by 2 washes with 0.2x SSC, 1% SDS, at 65°C. Hybridisation was quantified using a Phosphoimager.

cDNA was labelled by annealing *At4* specific primers to denatured cDNA template and synthesising new DNA strands using Klenow polymerase and (α^{32}P) dCTP.

2.3 DNA methods

2.3.1 PCR amplification of target sequences for cloning

Primers were designed to amplify target DNA sequences for cloning and to engineer enzyme sites for further cloning steps. Reactions were performed in a T3 thermocycler (Biometra) using High Fidelity PLATINUM® Taq DNA polymerase (GibcoBRL). PCR reactions were purified using Qiaquick spin kit (Qiagen).

2.3.2 One-step cloning of long, full-length PCR products

Long PCR products were cloned using the TOPO® cloning kit (Invitrogen). PCR products were incubated with the pCR®-XL-TOPO® plasmid vector, which was then transformed into One Shot® TOP 10 chemically competent cells. This was carried out according to manufacturers' instructions.

2.3.3 DNA digestion with restriction endonucleases

DNA digestion with restriction endonucleases was carried out according to enzyme manufacturers' recommended buffers and incubation temperatures.

2.3.4 Standard ligations

Ligations were performed using T4 DNA ligase (NEB) in the manufacturer's buffer and incubated as recommended. Target insert to vector DNA ratios were approximately 3:1.

2.3.5 Transformation of *Escherichia coli* competent cells

Chemically competent *E.coli* DH5α cells were prepared by inoculating overnight cultures into fresh LB broth (Sambrook *et al.*, 1989). Cells were grown to an OD_{600nm} between 0.5 and 0.6, after which they were spun down and resuspended in a half volume of 0.1M $MgCl_2$, followed by spinning down and resuspension in 1/20 volume of 0.1M $CaCl_2$ and 10% glycerol. All solutions were kept on ice. Transformations were carried out by adding plasmid DNA to 100 µl chemically competent *E.coli* DH5α cells at the moment of thawing, and incubated on ice for 30 min. Cells were heat-shocked at 42°C for 1 min., placed immediately on ice for 2 min., and then 1 ml of room temperature LB was added. Cells were incubated at 37°C, with shaking, for recovery. 100 µl cell

suspension was spread on selective LB plates. The remaining cell suspension was centrifuged and the majority of the supernatant was removed before being resuspended in the residual supernatant and spread on fresh, selective LB plates.

2.3.6 Agarose gel electrophoresis

DNA separation was performed on agarose gels made with and run in 0.5x TBE (45 mM Tris.HCl, 45 mM boric acid, 1 mM EDTA).

2.3.7 DNA isolation from agarose gels

Agarose gels were stained with ethidium bromide and visualised under UV light to distinguish different-sized DNA fragments. DNA fragments from restriction digests were isolated from agarose gels using Qiaex II gel extraction kit (Qiagen).

2.3.8 Isolation of *E.coli* plasmid DNA

Bacterial colonies were screened for correct plasmid insertions by inoculating 3 ml LB (containing the appropriate selective antibiotic) with single bacterial colonies and growing overnight at 37°C with shaking. Plasmid DNA was isolated from overnight cultures by alkaline lysis method as described by Sambrook *et al.* (1989). 1 ml culture was microfuged to collect bacterial pellet, the supernatant was poured off and the pellet was resuspended in the residual liquid. This was further resuspended in 250 µl buffer 1 (50 mM Tris, 100 mM EDTA, 400 µl RNaseA, pH 8). Cells were then lysed by adding 250 µl buffer 2 (200 mM NaOH, 1% SDS) and were left at room temperature for 5 min. The solution was neutralised by the addition of buffer 3 (2.55 M K acetate, pH 4.8) and mixed by gentle inversion. After centrifugation at 14000 rpm for 5 min., plasmid DNA was further purified by shaking with 200 µl phenol/ chloroform/ isoamylalcohol (25:24:1) mix and then centrifuging at 14000 rpm (Eppendorf centrifuge 5417C) for 5 min. The aqueous phase was removed, precipitated with one volume of isopropanol and then centrifuged at 14000 rpm (Eppendorf centrifuge 5417C) for 30 min. The supernatant was removed and the plasmid DNA pellet washed with 70% ethanol and centrifuged for 5 min. The supernatant was removed and the pellet air-dried then resuspended in 20 µl sterile H_2O.

Once a bacterial clone with the correct plasmid insert was identified, purified plasmid DNA was isolated from a culture of the chosen clone using Qiaprep mini-prep kit (Qiagen).

2.3.9 Transformation of *Agrobacterium tumefaciens*

Chemically competent *Agrobacterium* GV3101 cells were prepared by growing a single *Agrobacterium* colony overnight in 2 ml YEP media (10 g yeast extract, 10 g peptone and 5 g NaCl made up to 1 litre with H_2O, pH to 5.7 with NaOH). This was used to further inoculate 50 ml YE, which was then grown to 0.5 OD_{600nm}. Cells were then spun down and resuspended in 10 ml 0.15 M NaCl. Cells were again spun down and resuspended in 1 ml 20 mM ice-cold $CaCl_2$. Plasmid DNA were added to 100 µl chemically competent *Agrobacterium*, at moment of thawing, and incubated on ice for 30 min. Cells were frozen in liquid nitrogen, then immediately heat-shocked at 37°C until thawed. 1 ml of room temperature YEP was added. Cells were then incubated at 28°C, with shaking, for 2 – 4 h. The cell suspension was centrifuged and the majority of the supernatant was removed, before being resuspended in the residual supernatant and spread on fresh, selective YEP plates containing appropriate selective antibiotics.

2.3.10 Transformation of *Arabidopsis thaliana*

All *Arabidopsis* plants transformed with genetic constructs were in the Columbia background. Plants were transformed using a floral dipping method, as follows. Seedlings were grown under short day conditions (8 h light/ 16 h dark) for approximately 6 weeks, then transferred to long day conditions (16 h light/ 8 h dark) to induce flowering. Primary inflorescences were removed once they had reached 2–5 cm, to encourage growth of multiple secondary inflorescences. *Agrobacterium* cultures were prepared by inoculating 3 ml YEP (containing appropriate selective antibiotics) with one *Agrobacterium* colony containing the plasmid construct, and grown overnight at 28°C. This was used to inoculate 25 ml YEP (containing appropriate selective antibiotics), which was grown overnight. The 25 ml overnight culture was then used to inoculate a further overnight culture (500 ml). This was then centrifuged at 4200 rpm in a Beckman J6-MC for 20 minutes, the supernatant discarded, and the pellet resuspended in 0.5x MS solution to 1 litre. 200µl Silwet was added to the *Agrobacterium* solution. *Arabidopsis*

inflorescences were twice immersed in *Agrobacterium* solution. Trays of dipped *Arabidopsis* plants were placed in plastic bags overnight to encourage *Agrobacterium* infiltration.

2.3.11 Isolation of total *Agrobacterium* DNA

Agrobacterium DNA was isolated to determine whether any rearrangements of the plasmid construct had occurred. 1.5 ml of the *Agrobacterium* culture used for the transformation of *Arabidopsis,* was centrifuged (14000 rpm in an Eppendorf centrifuge 5417C for 1 min.). Pellets were resuspended in 300 µl TE. To lyse cells, 100 µl Sarkosyl in TE and 100 µl pronase in TE were added, and then incubated at 37°C for 2 h. The lysate was sheared by passing through a syringe. The aqueous phase was extracted twice with equal volumes of phenol saturated in TE. This was followed by extraction 2-3 times with equal volumes of chloroform. DNA was precipitated with 0.25 M NaCl and 2 volumes of ethanol at -20°C for 2 h. DNA was centrifuged for 5 min. at 14000 rpm. The supernatant was removed and the pellet was air-dried and then resuspended in 100 µl H_2O. Total *Agrobacterium* DNA was used to transform *E.coli* (only the plasmid DNA is incorporated). Plasmid DNA was isolated from *E.coli* and then digested with an enzyme that recognises multiple restriction sites in the plasmid. The resulting banding pattern was compared with that of the plasmid prior to *Agrobacterium* transformation.

2.4 Plasmid constructs for transformation of *Arabidopsis*

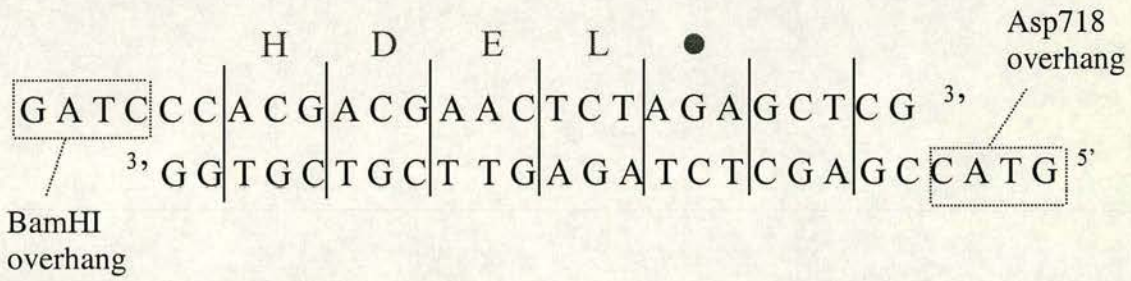
2.4.1 Generation of *At4* promoter::luciferase construct

A 956 bp flanking sequence of the *At4* gene representing the promoter region was PCR amplified from *Arabidopsis* Columbia genomic DNA using a specific 5' primer (GTCGACGATCTTGAAAGATTGTTGGGTG) to introduce a Sal I restriction site and a 3' specific primer (TCATGATTTGTGTGTTTGGTGTTGTGC) to introduce a BspH I site. The amplified fragment was cloned into the pCR-XL-TOPO vector (Invitrogen). The promoter fragment was released from pCR-XL-TOPO by digestion with BspH I and Spe I and transcriptionally fused to the luciferase reporter gene in the pSP-luc$^+$ vector (Promega), which had been cut with Nco I [BspH I compatible] and Avr II [Spe I compatible]. The *At4* promoter::luciferase fragment was released from the pSP-luc$^+$ vector by digestion with Sal I and Asp 718 and cloned into the pSPTV20 binary vector (cut with Sal I and Asp 718). The final plasmid construct was named pATSL and was used to transform Columbia-background *Arabidopsis* plants. Plants transformed with this construct were not included in the work of this thesis but a mutagenised population of *At4* promoter::luciferase plants will be used in future to screen for plants carrying mutations in the phosphate response.

2.4.2 Generation of *At4* promoter::GFP construct

Compatible restriction sites were engineered into the mGFP6 gene by PCR amplification of mGFP6 using a 5' specific primer (GGAACTACAAGACACGTGCTGAAG) to introduce a BspH I restriction site and an Xba I restriction site, and using a 3' specific primer (TCCCTTAAGCTCGATCCTGTTG) to introduce a Kpn I site, a Sac I site, a BamH I site and a STOP codon. The amplified fragment was cloned into the pGEMTeasy vector (Promega). The GFP6 gene was released from the pGEMTeasy vector by digesting with Asp 718 and BspH I. The 956 bp *At4* promoter fragment was released from the pCR-XL-TOPO vector (see section 2.4.1) by digestion with BspH I and Sal I. A three-way cloning was performed with the GFP6 fragment, the *At4* promoter fragment and a pBluescript KS$^-$ vector (Stratagene) digested with Asp 718 and Sal I. This resulted in a transcriptional fusion of the *At4* promoter with the GFP6 gene. The *At4*promoter::GFP fragment was released from the pBluescript KS$^-$ vector by

digestion with Asp718 and PstI and cloned into the pGEM7 vector (Promega) cut with Asp 718 and Nsi 1 [Pst I compatible]. An ER retention signal (HDEL) was cloned into the 3' end of the GFP6 gene. Two oligonucleotides were annealed to create the ER retention signal with Bam HI and Asp718 overhangs (see below). Annealling was performed by adding 10 µl of each oligo (10 µg/µl) together and denaturing at 80°C for 5 min. The oligos were allowed to cool slowly at room temperature to anneal.



A 1:10 dilution of the annealed ER retention signal was cloned into the pGEM7 *At4* promoter::GFP construct (digested with Asp 718 and BamH I). The *At4* promoter::GFP fragment containing ER retention signal was released from the pGEM7 vector by digestion with Asp 718 and Sal I and cloned into the pGPTV20 binary vector (cut with Asp 718 and BamH I). The final plasmid construct was named pATSG, which was used to transform *Agrobacterium.* One *Agrobacterium* colony containing this plasmid was used to transform *Arabidopsis* plants in the Columbia background. Seeds were collected from these plants and plated on 1 x MS medium containing 15 µg/ ml Kanamycin. Kanamycin- resistant seedlings were transferred to soil. Seeds were collected from individual plants and plated on 1 x MS medium containing 15 µg/ ml Kanamycin. Kanamycin –resistant seedlings from those displaying 3:1 segregation were transferred to soil. Seeds from these individual plants were again plated on selection medium. Several plants with all progeny homozygous for Kanamycin resistance were tested for strength of GFP expression under Pi starvation and one line showing strong GFP expression was chosen for further experiments.

2.4.3 Generation of 35S::*At4* construct

A 709 bp fragment representing the *At4* gene was PCR amplified from Arabidopsis Columbia genomic DNA using a specific 5' primer (CTCTAGACAAAGAGAGAAGC CATAAAAACCC) to introduce an Xba I restriction site and also using a 3' specific primer (CGAGCTCTTAATGAAAGGTAGAAGTGGGG) to introduce a Sac I site. The amplified PCR product was cloned into the pGEMTeasy vector (Promega). The *At4* gene was released from the pGEMTeasy vector by digesting with Xba I and Sac I and was transcriptionally fused with the 35S promoter in the 35S-TL2 pGEM5 vector in a methylation minus background (cut with Xba I and Sac I). The 35S::*At4* fragment was released from the 35S-TL2 pGEM5 vector by digesting with Spe I and Sac I and was cloned into the pGPTV20 binary vector (cut with Xba I [Spe I compatible] and Sac I). The final plasmid construct was named p35SAT pATSG, which was used to transform *Agrobacterium*. One *Agrobacterium* colony containing this plasmid was used to transform *Arabidopsis* plants in the Columbia background. Seeds were collected from these plants and plated on 1 x MS medium containing 15 μg/ ml Kanamycin. Kanamycin- resistant seedlings were transferred to soil. Seeds were collected from individual plants and plated on 1 x MS medium containing 15 μg/ ml Kanamycin. Kanamycin –resistant seedlings from those displaying 3:1 segregation were transferred to soil. Seeds from these individual plants were again plated on selection medium. Several plants with all progeny homozygous for Kanamycin resistance were tested for strength of At4 over-expression and one line with the highest At4 over-expression was chosen for further experiments.

2.5 *Arabidopsis* mutants

2.5.1 *pho1* mutant

The *pho1* mutant was first identified by Poirier et al. (1991) during a screen of EMS-mutagenised Arabidopsis Columbia plants for mutants containing altered levels of total leaf phosphate. The mutant was found to accumulate only 5% of wild-type Pi levels as a result of a single nuclear recessive mutation at the *pho1* locus. PHO1 was later found to be located in the pericycle and xylem parenchyma cells (Hamburger et al., 2002). PHO1 is believed to mediate the efflux of Pi from root stelar cells for loading into the xylem stream. The mutant displays many phosphate-deficient characteristics including small leaves, thin stalks, delayed flowering and reduced germination frequency. The *pho1* mutant was kindly supplied by Yves Poirier.

2.5.2 *pho2* mutant

The *pho2* mutant was discovered by Delhaize and Randall (1995) during a screen for phosphate-accumulating mutants from a mutagenised population of *Arabidopsis* seedlings (Columbia ecotype). The *pho2* mutant is a recessive, single-gene mutant that accumulates 2 to 5 fold more Pi in its leaves compared to wild-type plants. Visible defects are only evident during high transpiration rates, when leaves become necrotic. The *PHO2* gene has yet to be cloned but it is thought to either function in phloem transport or to have a role in Pi-sensing. The *pho2* was supplied by Emmanuel Delhaize.

2.5.3 *phr1-1* mutant

The *phr1-1* mutant was isolated by Rubio *et al.* (2001) during a screen of an EMS-mutagenised *Arabidopsis* (Columbia ecotype) population harbouring a reporter gene (AtIPS1::GUS). The *phr1-1* mutant was identified by its reduced GUS staining during Pi starvation conditions. This mutant also has impaired Pi starvation responses, including the reduced expression of Pi starvation response genes during Pi limitation. The *PHR1* gene encodes a member of the MYB superfamily that is conserved between *Arabidopsis thaliana* and *Chlamydomonas reinhardtii.* PHR1 is thought to act downstream in the phosphate signalling pathway. The *phr1-1* mutant was supplied by Javier Paz-Ares.

2.5.4 *cre1-1* mutant

Cre1 mutants were first identified by Franco-Zorilla *et al.* (2002) during a screen of an EMS-mutagenised *Arabidopsis* (Columbia ecotype) population harbouring a reporter gene (AtIPS1::GUS). The mutagenised population were grown in Pi starvation conditions in the presence of 10μM kinetin and *cre1* mutants were found to display impaired kinetin repression of AtIPS1::GUS activity. These lines were also found to have impaired kinetin repression of other Pi starvation response genes and to have reduced anthocyanin accumulation during Pi starvation. The *cre1-1* mutant in the Landsberg erecta background was supplied by Antonia Leyva.

CHAPTER 3: MORPHOLOGICAL, PHYSIOLOGICAL AND MOLECULAR RESPONSES OF *ARABIDOPSIS* TO PHOSPHATE AVAILABILITY

3.1 Summary

Plants mount many responses to phosphate limitation. The aim of this chapter was to compare the kinetics of each response. Pi starvation responses representing morphological, physiological and molecular alterations of *Arabidopsis* seedlings in response to Pi deprivation and Pi re-supply were determined. Starvation responses affecting growth, henceforth called morphological responses, were found to occur later than molecular and physiological responses. Many alterations in gene expression and physiology are induced in order to scavenge internal and external Pi reserves. Growth responses appear to occur after extended periods of Pi starvation, perhaps once Pi reserves have been utilised. It is also possible that methods to quantify growth were less sensitive than the molecular techniques used to detect the other responses. The extent of all responses was found to be dependent on the length of Pi starvation and on the previous Pi growth conditions. Furthermore, the speed of recovery of gene expression to basal expression levels, after re-supply of phosphate, indicated that local Pi concentration controls molecular and physiological responses.

3.2 Introduction

Plant responses to phosphate limitation fall into three main categories: morphological, physiological and molecular. Visible morphological alterations that occur in plants experiencing Pi starvation include increases in dry weight root-to-shoot ratios (Trull *et al.*, 1997, López-Bucio *et al.*, 2002). Lateral root initiation and elongation is also enhanced, whilst primary root length decreases (Williamson *et al.*, 2001; Linkohr *et al.*, 2002; López-Bucio *et al.*, 2002). In addition, root hairs become more dense and elongated (Fohse *et al.*, 1991; Bates and Lynch, 1996; Ma *et al.*, 2001). Recorded physiological changes include reduced rates of photosynthesis and carbon fixation (Härtel *et al.*, 1998; Hurry *et al.*, 2000; Pieters *et al.*, 2000; Poirier and Bucher, 2002). Membrane lipid composition is also altered under Pi stress as galactolipids and

sulfolipids replace phospholipids. Key genes underpinning these processes have been found to be transcriptionally-regulated in response to Pi availability. These include *MGD3* and *SQD1*, which are necessary for the biosynthesis of galactolipids and sulfolipids, respectively (Essigmann *et al.*, 1998; Härtel *et al.*, 2000; Klaus *et al.*, 2002; Yu *et al.*, 2002). Furthermore, alternative glycolytic and respiratory pathways are utilised by Pi starved plants to circumvent high Pi-requiring steps (Duff *et al.*, 1989; Theodoru and Plaxton, 1993). Currently known molecular responses in *Arabidopsis* include the enhanced expression of genes encoding: high-affinity Pi-transporters such as *Pht1;1*, *Pht1;2*, *Pht1;3* and *Pht1;4* (Muchal *et al.*, 1996; Smith *et al.*, 1997, Okumura *et al.*, 1998; Dong *et al.*, 1999; Mudge *et al.*, 2002), acid phosphatases such as *AtACP5* (del Pozo *et al.*, 1999) and ribonucleases such as *RNS1* and *RNS2* (Bariola *et al.*, 1994). The following genes of unknown function are also induced by Pi stress, *At4* (Burleigh and Harrison, 1997), *AtIPS1* (Martin *et al.*, 2000) and *PSR3.2* (Malboobi and Lefebvre, 1997). A recent micro-array experiment has identified many genes that are up-regulated in *Arabidopsis* shoots after Pi withdrawal. The functions of these genes were extremely varied. Of the 60 genes that were transiently induced after 4 hours of starvation, most had mainly metabolic or cell rescue and defence roles, whereas the 49 genes that were expressed later (after 100 h) were mostly Pi-starvation specific or were involved in Pi metabolism (Hammond *et al.*, 2003).

The signalling pathways regulating plant phosphate starvation responses are as yet unknown. Different responses may be under different control. The expression of some Pi-inducible genes has been found to be under the control of a shoot-derived systemic signal (Liu *et al.*, 1998b; Burleigh and Harrison, 1999), whereas root hair density and elongation have been shown to be under the control of local Pi availability (Bates and Lynch, 1996).

The majority of phosphate starvation responses have been studied individually. However, it has become increasingly evident that it is the coordination of all these responses that allow plants to survive phosphate limitation. Therefore, comparative studies of all response types should be conducted to provide insights into how whole plants mount responses once Pi starvation is perceived. This may shed light on whether all responses are initiated simultaneously or whether there is a hierarchy of activation. In addition, most responses have been characterised at only one time interval after a period of phosphate starvation and this time-point can vary enormously between different

researchers. Experimental conditions can also be extremely variable and this too can make comparisons difficult.

Therefore, the aim of the experiments reported in this chapter, is to study the kinetics of the different phosphate-starvation response categories under standardised experimental conditions. Primary root length and primary root cortical cell length will act as indicators of morphological change. Molecular responses will be represented by the analysis of *Pht1;1*, *At4*, *AtIPS1* and *AtACP5* gene expression. The small scale of these experiments prevents analysis of lipid composition and enzyme activity. Therefore, indicators of physiological change will include *MGD3* and *SQD1* gene expression. The timing of these different responses will be compared in plants experiencing different Pi availability and in plants transferred to Pi starvation conditions.

3.3 Results

3.3.1 *Arabidopsis* growth responses to phosphate availability

The effect of different Pi availability on *Arabidopsis* root growth was determined Seedlings were grown on vertically oriented plates containing a range of phosphate concentrations. The progression of primary root growth was measured daily (Fig. 3.1).

Figure 3.1a shows root length of seedlings grown in low Pi conditions (0 to 62.5 μM Pi). No difference in root length was observed between plants grown in different Pi conditions in the first 4 days. However, after six to seven days, primary root length was significantly shorter with decreasing Pi concentration. Root growth rates of seedlings grown in low Pi indicate that primary root growth was already slower in lower Pi concentrations between day 4 and day 5 (Fig 3.1c). Root growth rate of seedlings grown at all low Pi concentrations decreased over time before finally reaching a slow, continuous, steady state of growth. The rate and timing of this response was dependent on the Pi condition, with the conversion from decreasing root growth to slow, invariable growth beginning from day 7 for no Pi, day 8 for 20 μM Pi, day 10 for 40 μM Pi and day 11 for 62.5 μM Pi. The rate of uniform growth was also dependent on Pi availability, from 0.4 mm/day for no Pi to 1.7 mm/ day for 62.5 μM Pi.

Under higher phosphate conditions (125 μM to 2.5 mM Pi), primary root length was similar for seedlings grown at all concentrations until day 7 (Fig 3.1b). After day 7, increases in Pi concentration resulted in longer root length. Figure 3.1d shows the corresponding growth rates. Root growth rate remained relatively unchanged at 125 μM Pi over time but increased with time in the higher Pi concentrations. This increase in growth rate was also dependent on Pi availability, although there was no difference between the two highest Pi concentrations of 1 mM and 2.5 mM Pi.

The effect of Pi availability on cortical cell length in primary roots was determined by growing *Arabidopsis* Q8 PIP2A seedlings in vertical plates containing different Pi concentrations. Cell membrane-localisation of GFP in Q8 PIP2A seedlings facilitated accurate measurements of cortical cell size (Cutler *et al.*, 2000). The length of primary roots was compared to cortical cell length after 5 and 11 days growth. There was little difference in primary root length between different Pi conditions after 5 days (Fig 3.2a). However, there was a substantial difference after 11 days, with root length at 2.5 mM Pi almost double that at no Pi. There was similarly no difference in cortical cell length on

day 5, with mean cortical cell length ranging from 135 µm at 125 µM Pi to 150 µm at no Pi and at 2.5 mM Pi (Fig 3.2b). By day 11, root cortical cell length had decreased to 90 µm at no Pi, there was no change at 125 µM Pi and there was an increase to 202 µm at 2.5 mM Pi. Plants grown in higher Pi availability have longer primary roots and this correlates with an increase in the length of root cortical cells. Longer primary roots found in seedlings grown under high Pi availability are therefore at least partly due to an increase in root cell size.

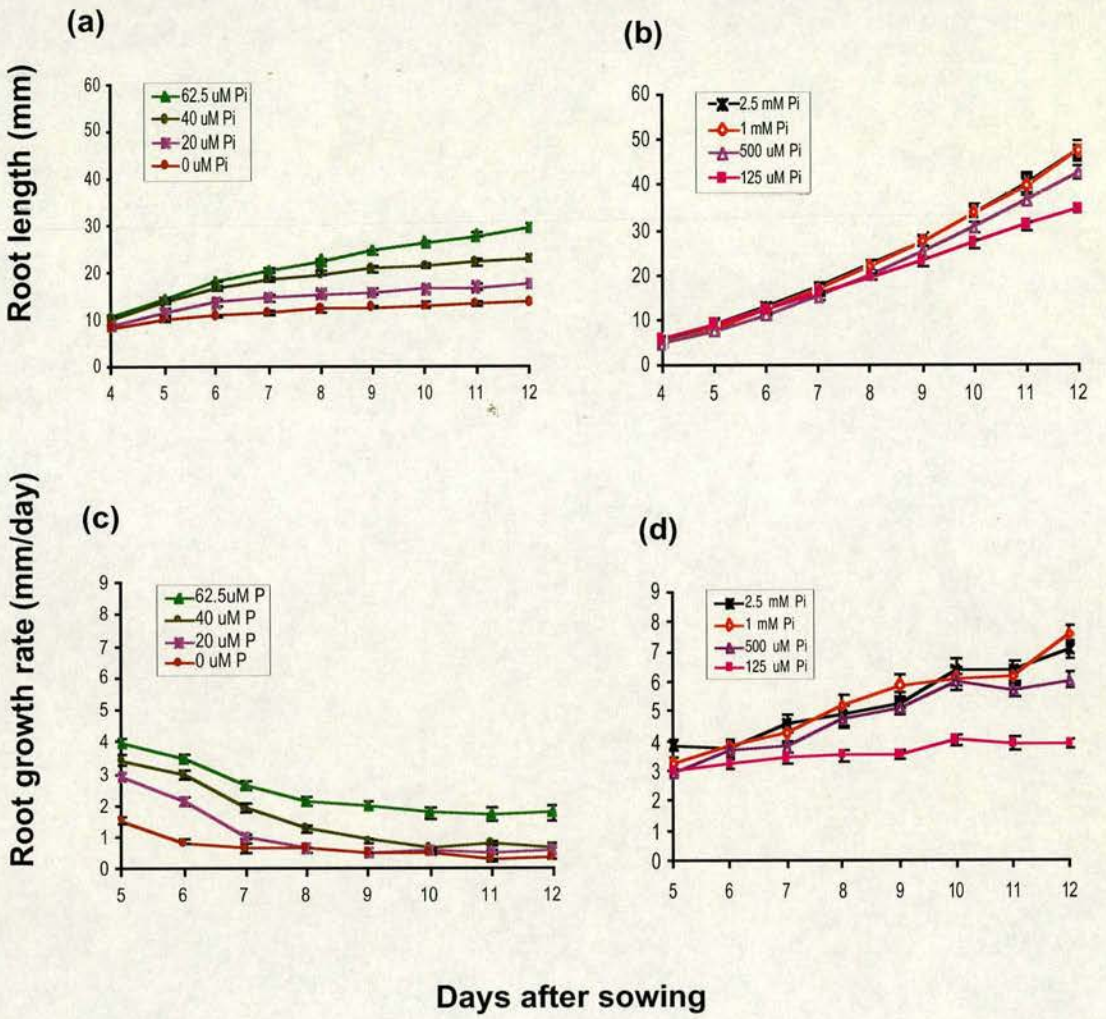


Figure 3.1: The effect of phosphate concentration on primary root growth. *Arabidopsis* Col0 seedlings were grown on vertical plates in 0.5x MS media containing different Pi concentrations. Root length, measured every 24 h, is depicted for a) low Pi and b) high Pi. Growth rates of the same primary roots over the previous 24 h were calculated for c) low Pi and d) high Pi. Values represent mean (+/- SE) of 25-35 seedlings. Data represent one of three independent repeat experiments.

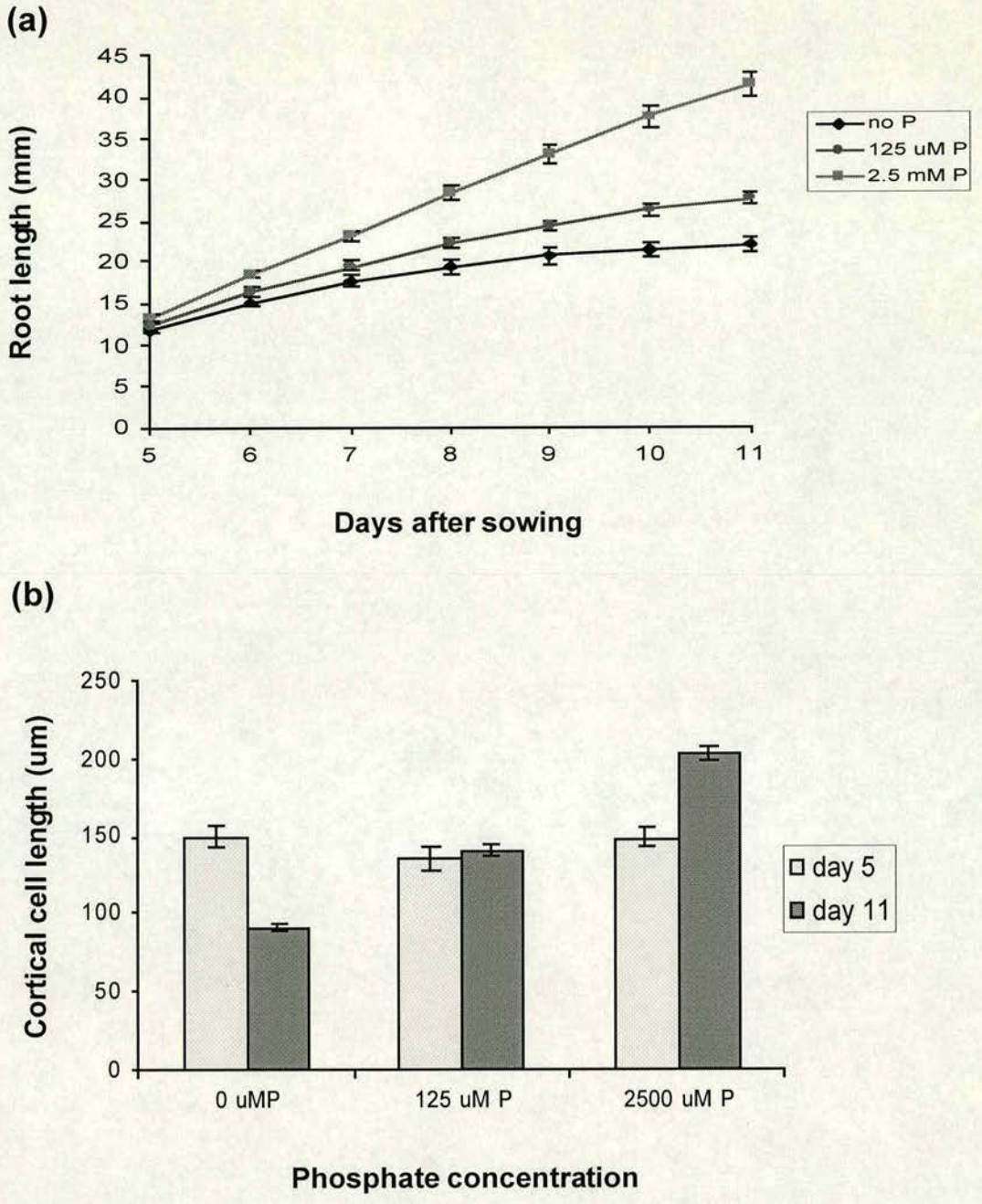


Figure 3.2: Root length versus cortical cell length of *Arabidopsis* Q8 PIP2A seedlings grown in 0.5x MS at different Pi concentrations. Graphs show (a) primary root length over time, values represent means of 15-25 seedlings (+/- SE) and (b) cortical cell length at 5 and 11 days after sowing, values represent mean cell length (+/- SE) pooled from 6 different primary root samples. From each root, 15 cortical cells located approximately 0.5 cm above the elongation zone, were measured. Data represent one of three independent repeat experiments.

3.3.2 Molecular and physiological responses to phosphate availability

The expression of several *Arabidopsis* Pi starvation-inducible genes under different Pi conditions was analysed to discover when molecular and physiological responses are initiated. Seedlings were grown in vertical plates containing different Pi media for 5, 7 and 11 days (from which growth data in figure 3.2 was obtained). Gene expression in root tissue was determined by Quantitative RT-PCR analysis. Data obtained for day 11 was found to be inconclusive and irreproducible. Many seedlings grown for 11 days in no Pi conditions appeared extremely physically stressed (and possibly dead) which would almost certainly have had an effect on gene expression. In addition, a substantial amount of Pi collected at the bottom of vertical plates over longer periods of time thus reducing the homogenous distribution of Pi within the media.

Nonetheless, it can be seen from Figure 3.3 that there was a substantial difference in gene expression between seedlings grown on different Pi conditions after only 5 days. This was the case for all genes tested. Expression of phosphate starvation response genes was expected to be low in seedlings grown at 2.5 mM Pi and this was taken as the basal level of expression. Expression at all other Pi concentrations was calculated as a fold-increase relative to basal expression. After 5 days, expression of most genes was slightly higher in roots of plants grown at 125 μM Pi than at 2.5 mM Pi. Gene expression increased further after 7 days in 125 μM Pi. *At4* was the only gene to be highly induced at this Pi concentration, with 110-fold increase in expression after 7 days (Fig. 3.3b). All genes were expressed at even higher levels in plants grown in no Pi conditions and expression increased further after 7 days, by as much as 6-fold in the case of *AtIPS1* (Fig.3.3c). *Pht1;1* was the exception, with lower expression levels after 7 days.

The observed variation in expression indicates that there is no threshold value of Pi starvation at which these genes are switched either on or off. Rather, there is a graded response to Pi concentration i.e. gene expression increases accordingly with decreasing Pi concentration and with increased exposure to Pi limiting conditions. Fold induction of gene expression in response to Pi starvation was markedly different for each gene studied. *Pht1;1* increased to no more than 4 fold that of basal level, whereas after 7 days in no Pi media, *At4* and *AtIPS1* expression had increased 200 and 150 fold, respectively. Under the same conditions, *MGD3* was induced roughly 30-fold and *SQD1*only 12 fold.

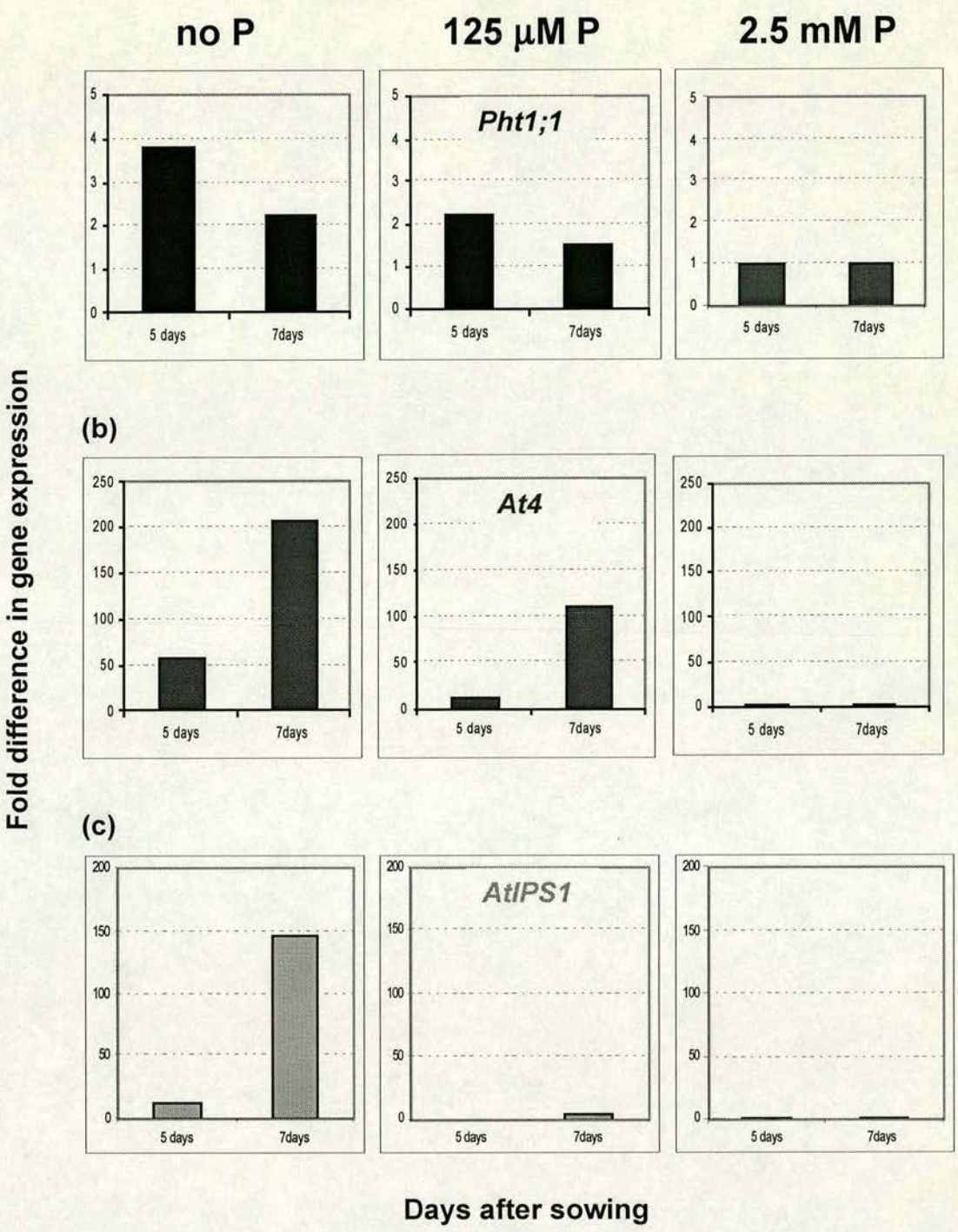


Figure 3.3: Expression of phosphate starvation response genes: a) *Pht1;1* b) *At4* and c) *AtIPS1*, in roots of *Arabidopsis* Col0 seedlings. Root tissue was sampled 5 and 7 days after seeds were sown on 0.5x MS vertical plates containing no Pi, low Pi (125 µM) or high Pi (2.5 mM). Fold difference in expression, determined by Quantitative RT-PCR, is relative to expression of 2.5 mM P, 5 day sample and has been normalised for cDNA loading by comparing threshold cycles of amplification of the gene of interest to that of the constitutively expressed *eIF4A* gene. Data represent one of two independent repeat experiments.

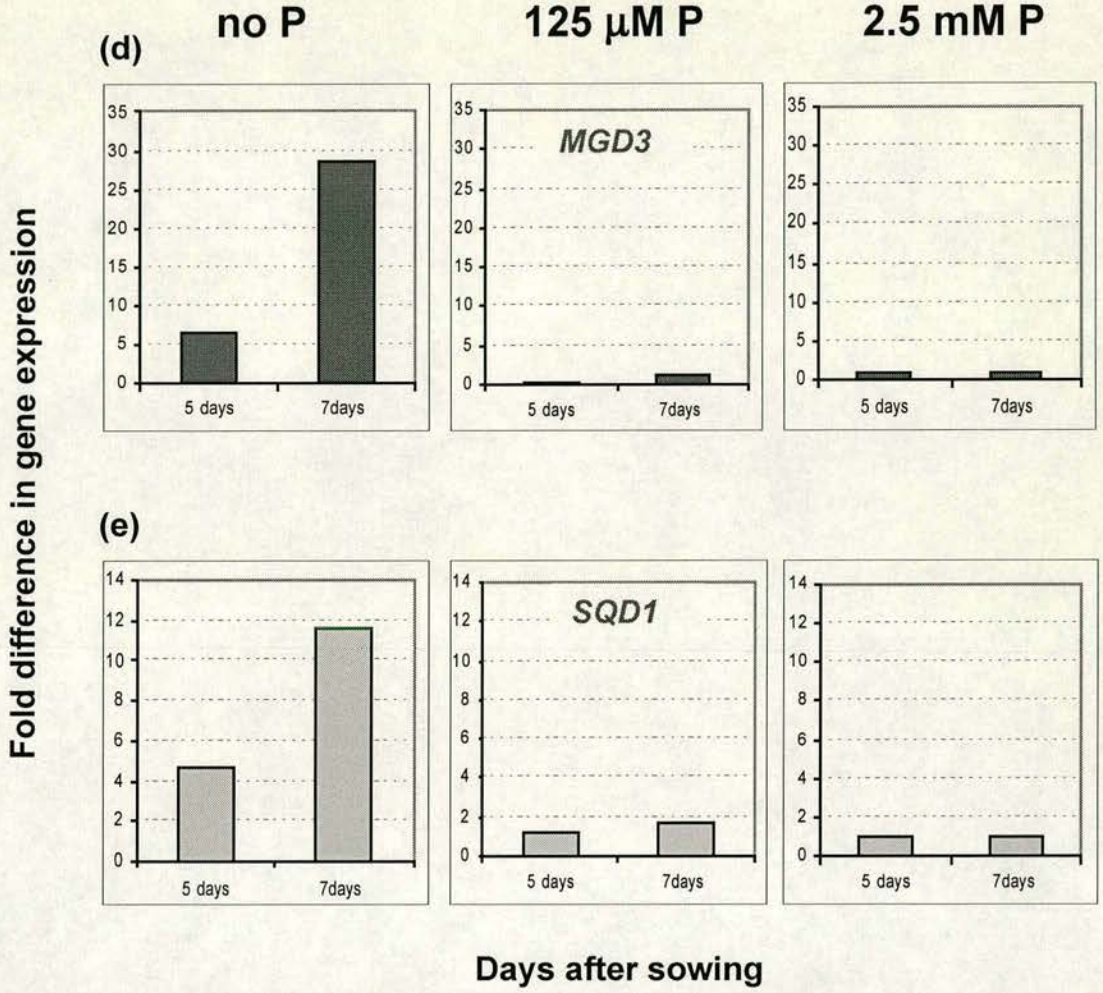


Figure 3.3 (continued): Expression of phosphate starvation response genes: d) *MGD3* and e) *SQD1* in roots of *Arabidopsis* Col0 seedlings. Root tissue was sampled 5 and 7 days after seeds were sown on 0.5x MS vertical plates containing no Pi, low Pi (125 µM) or high Pi (2.5 mM). Fold difference in expression, determined by Quantitative RT-PCR, is relative to expression of 2.5 mM P, 5 day sample and has been normalised for cDNA loading by comparing threshold cycles of amplification of the gene of interest to that of the constitutively expressed *eIF4A* gene. Data represent one of two independent repeat experiments.

3.3.3 Growth response kinetics after transfer to starvation conditions

The growth response kinetics of *Arabidopsis* seedlings transferred to Pi-starvation conditions, was investigated. Seedlings were grown on vertical plates supplied with different concentrations of phosphate and then transferred to fresh vertical plates

containing no Pi or the pre-growth Pi condition. Primary root growth prior to, and after transfer, was measured.

Primary root growth rate of seedlings pre-grown in 125 μM Pi was steady prior to transfer (Fig 3.4a). Control seedlings transferred to 125 μM Pi displayed initially slower growth rates than during pre-transfer but steadily increased back to pre-transfer levels by 48 h after transfer (AT). Root growth then continued at the same steady rate (~ 1.8 mm / 12 h). Seedlings transferred to no Pi also grew at an initially slower rate after transfer but, unlike the control, maintained this rate until 48 h AT. Root growth rate then declined until 100 h AT and was thereafter maintained at a slow, relatively steady rate (~ 0.7 mm / 12 h).

Seedlings pre-grown at 62.5 μM Pi displayed declining root growth prior to transfer (Fig 3.4b and repeat experiments). Again, root growth rate of control seedlings transferred to 62.5 μM P was slower immediately after transfer, although it steadily increased back to pre-growth levels after approximately 48 h AT. Root growth rate then declined (continuing on the slope of pre-growth rate decline) until around 150 h AT and was subsequently maintained at a slow, but steady, rate (~ 0.8 mm / 12 h). Root growth rate of seedlings transferred to no Pi was similar to that of control seedlings 12 h AT but, unlike the control, growth rate did not increase after this point. Instead, it remained constant until 48 h AT, after which point it began to decline. The decline halted at approximately 100 h AT. Root growth rate then remained constant at a slower rate than control seedlings (~ 0.5 mm / 12 h).

There were therefore 3 distinct phases of primary root growth after seedlings were transferred to no Pi conditions. Firstly, there was a 'lag period' immediately after transfer, lasting until approximately 48 h AT, during which signalling in response to either external or internal Pi status is presumably initiated. Over this period, root growth rate of control seedlings transferred to pre-transfer Pi concentrations increased back to pre-transfer levels. However, seedlings that were transferred to no Pi did not immediately respond to external Pi status by decreasing their growth rate and instead maintained a constant growth rate. The second phase of growth occurred between 48 – 100 h AT. During this time there was a continuous decline in root growth rate as plants responded to lower Pi levels. By 100 h AT, the decline had halted and the third phase of growth, the adjustment of growth to a slow, steady rate, began. Plants transferred from

62.5 μM P back to 62.5 μM P also showed declining growth rates followed by a slow, steady rate but the timing of entering these phases was later and the final slow, steady rate was slightly higher than those transferred to no Pi. This confirms that 62.5 μM Pi is limiting to plant growth but it also confirms that plants adjust the kinetics of their growth and metabolism according to the level of Pi availability and duration of Pi limitation.

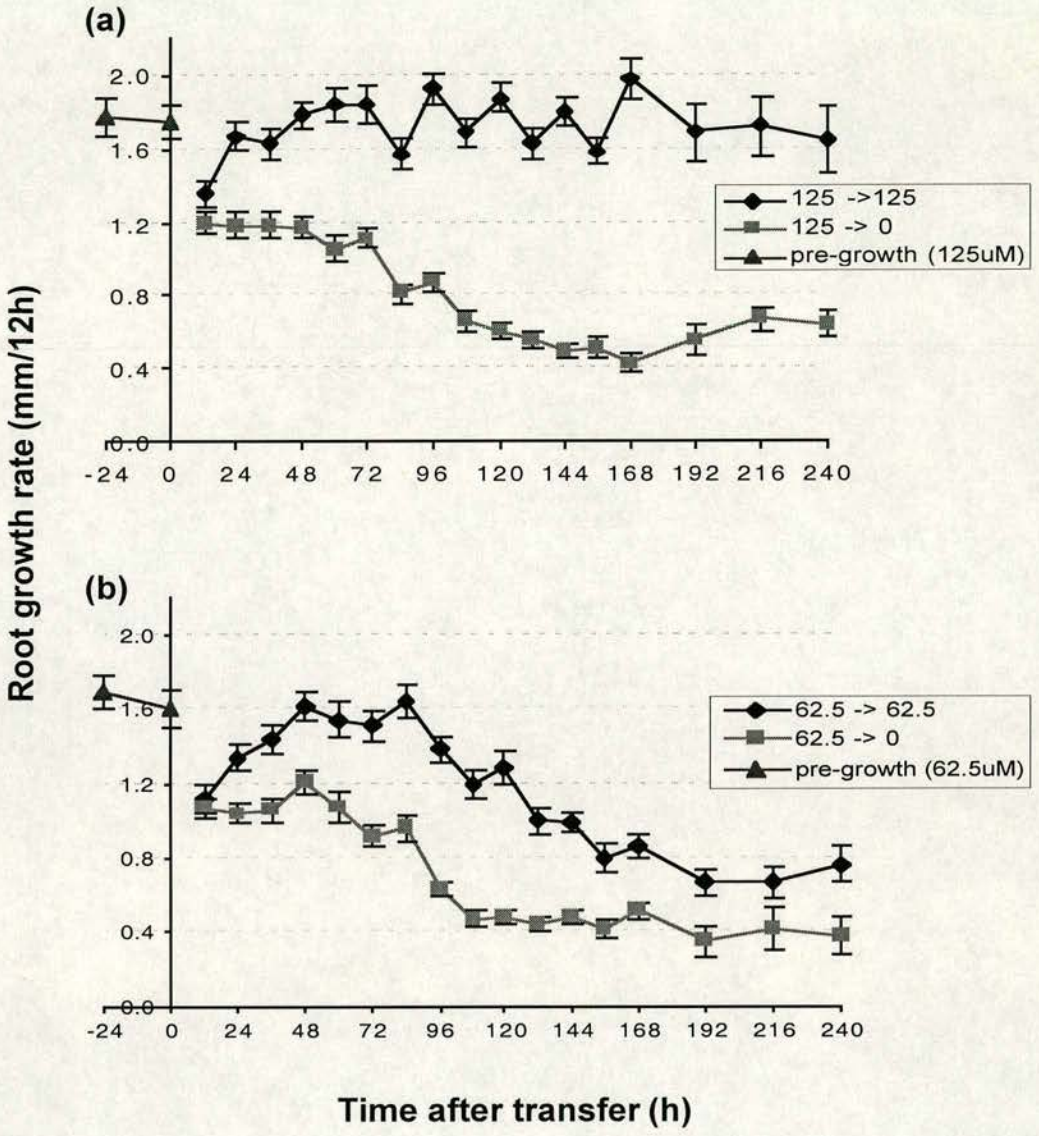


Figure 3.4: Root growth rate of *Arabidopsis* Col 0 after transfer to no Pi media. Seedlings were pre-grown for 7 days on vertical plates in 0.5x MS media containing a) 125 μM Pi or b) 62.5 μM Pi, then transferred to 0.5x MS media containing no Pi or to the same Pi condition as during pre-growth. Data represent mean growth (+/- SE) of 30-35 individual primary roots over 12 h. Data represent one of two individual repeat experiments.

3.3.4 Molecular and physiological response kinetics after transfer to phosphate starvation conditions

To gain further insight into the timing of phosphate perception and signalling, the kinetics of gene expression after transfer to phosphate starvation was assessed. *Arabidopsis* seedlings were pre-grown in flasks containing low and high Pi liquid media. Seedlings were then transferred to Pi starvation conditions. Root tissue was sampled from plants grown for different periods of starvation and Quantitative RT-PCR analysis was performed to determine gene expression. Fold increases in expression are relative to expression in roots of seedlings pre-grown at 2.5 mM on transfer to Pi starvation (t=0).

From steady state experiments (sections 3.3.1 and 3.3.2) it became clear that molecular and physiological responses occur prior to any major difference in root growth under low Pi conditions. Therefore, an early induction of Pi starvation response genes was expected on transfer to no Pi, hence the detailed kinetics for the first 24 h of Pi starvation (Fig 3.5). For each gene tested, there was little induction within this initial starvation period. Only at the 18 to 24 h AT (after transfer) stage was there any increase in gene expression. The one anomaly in this data set was a transient increase of *Pht1;1* expression 3 h after transfer to starvation conditions from 2.5 mM Pi. A 5-fold induction of *Pht1;1* expression is relatively high considering that there is only a 4-fold induction under the same conditions after 120 h of starvation. Unfortunately, the equivalent 3 h RNA sample for 125 μM Pi pre-grown plants was degraded and could not be subjected to RT-PCR. Further experiments to corroborate this early *Pht1;1* regulation are presented in section 3.3.5.

There were substantial increases in gene expression 48 h AT in roots of plants pre-grown at 125 μM Pi and expression steadily increased throughout the duration of the experiment. The one exception to this was *AtACP5* (Fig 3.5b) which seemed to peak at 48 h, proceeded by a decrease in expression levels. However, repeat experiments did not recreate this pattern of expression and instead *AtACP5* expression was similar to that of the other genes represented (data not shown). Seedlings that were pre-grown in high Pi conditions (2.5 mM) displayed slower induction of Pi-starvation response genes. In particular, *Pht1;1*, *At4* and *MGD3* expression was not induced until as late as 96 h AT. Not only was the response later but the level of induction was also considerably less.

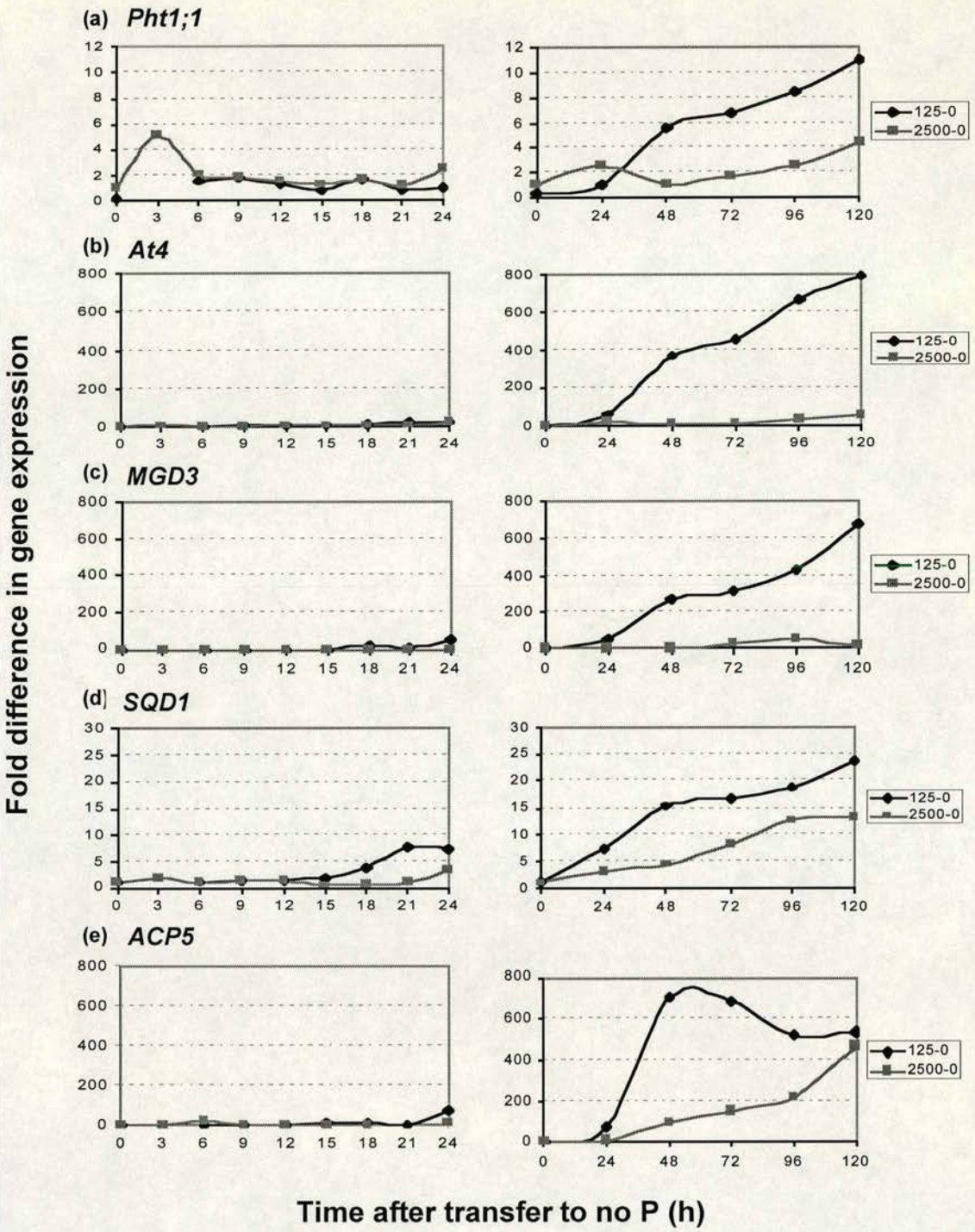


Figure 3.5: Expression kinetics of phosphate starvation response genes: a) *Pht1:1*, b) *At4*, c) *MGD3*, d) *SQD1* and e) *ACP5* in *Arabidopsis* Col0 roots after transfer to no Pi media. Seedlings were pre-grown in liquid 0.5x MS media containing either low Pi (125 µM) or high Pi (2.5 mM). Fold difference in expression, determined by Quantitative RT-PCR, is relative to expression of 2.5 mM Pi t=0 sample and has been normalised for cDNA loading by comparing threshold cycles of amplification of the gene of interest to that of the constitutively expressed *eIF4A* gene. Data represent one of two individual repeat experiments.

This also proves that the level of gene induction in response to starvation is dependent on the strength of the starvation signal, which is in turn dependent on the Pi status of the plant.

3.3.5 *Pht1;1* expression kinetics after transfer to phosphate starvation conditions

In section 3.3.4, *Pht1;1* expression transiently increased 3 h after transfer to media containing no Pi. Many experiments, using the same method, were conducted to prove its repeatability. In all cases there was found to be a peak of *Pht1;1* expression after 2 h although the amplitude of the peak ranged from 4 to 12 fold that of t=0 expression.

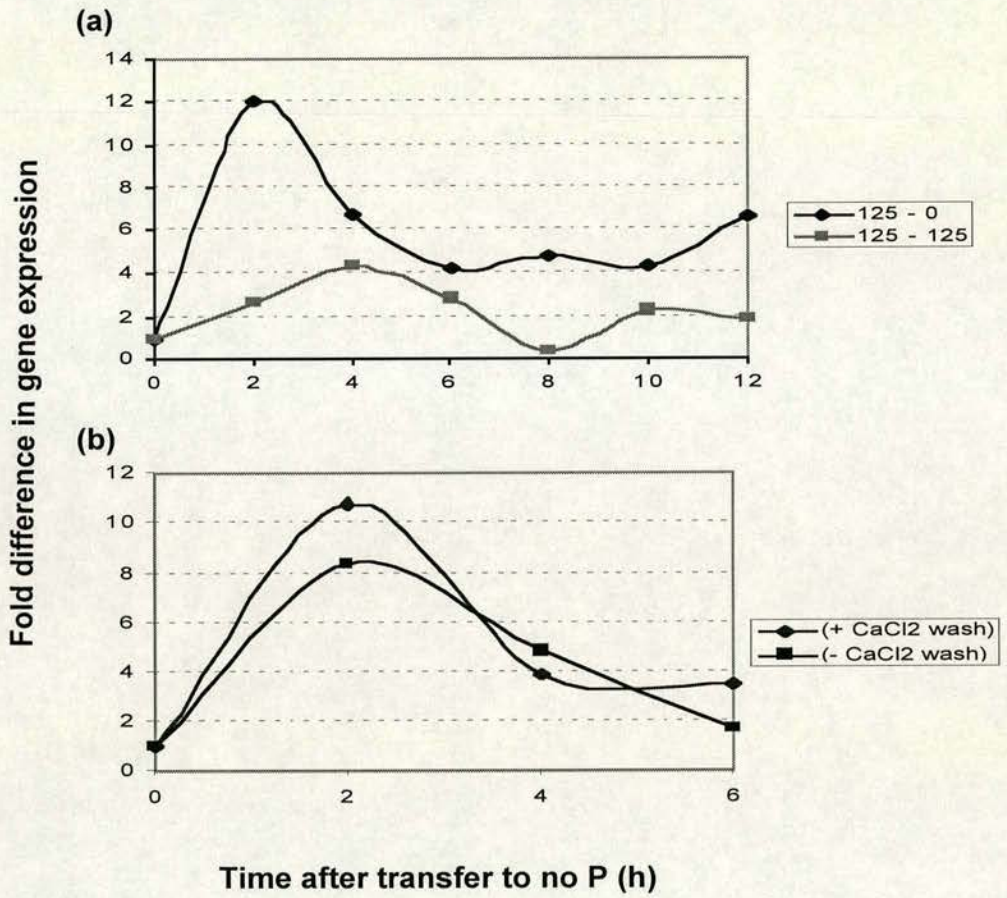


Figure 3.6: *Pht1;1* expression kinetics in *Arabidopsis* Col0 roots after seedlings, pre-grown in 0.5x MS liquid media containing 125 µM Pi, were transferred to: a) no Pi media or 125 µM Pi media and b) no Pi media, with or without a calcium chloride wash prior to transfer. Fold difference in expression, determined by Quantitative RT-PCR, is relative to expression of t=0 sample and has been normalised for cDNA loading by comparing threshold cycles of amplification of the gene of interest to that of the constitutively expressed *eIF4A* gene. Data represent two of several independent repeat experiments.

Figure 3.6a shows a peak of *Pht1;1* expression 2 h AT in roots of seedlings transferred to starvation conditions. Control plants that were transferred back to the pre-growth Pi concentration did not display this response. This proves that the transfer process itself was not responsible for the peak of *Pht1;1* expression. Calcium chloride treatment was used to remove Pi from roots. This was ruled out as a possible reason for *Pht1;1* induction as there is still a peak of *Pht1;1* expression in roots of untreated plants (Fig. 3.6b). The amplitude of the peak was slightly lower than the peak seen in plants washed with $CaCl_2$. This is likely because more phosphate was removed from the roots of $CaCl_2$ – washed plants (the purpose for which it was originally applied).

Therefore, *Pht1;1* expression peaks transiently in plants two hours after transfer to phosphate starvation. This response seems too rapid to be under the control of a systemic signal, suggesting that *Pht1;1* may be under local control.

3.3.6 Kinetics of recovery from phosphate starvation

The timing of phosphate starvation responses has been found to be dependent on the pre-starvation Pi concentration. It is assumed from these results that Pi signalling and subsequent starvation responses are delayed by the presence of greater internal Pi stores in seedlings pre-grown at higher Pi concentrations (see sections 3.3.3 and 3.3.4). Internal Pi stores may delay signalling despite decreased Pi availability in the environment. Re-supplying Pi to starved plants ensures that responses will not be shielded by the presence of Pi stores. It should provide a more precise indication of immediate Pi signalling kinetics since there should be no delay for scavenging and re-allocation of stores. *Arabidopsis* seedlings were pre-grown in 500 μM Pi, transferred to starvation conditions for 5 days, then re-supplied with 2.5 mM Pi. Quantitative RT-PCR analysis was performed on root tissue sampled from seedlings at different time intervals after re-supply. Fold increases in expression are relative to root expression of seedlings on transfer to Pi starvation.

Graphs in figure 3.7 reveal that recovery of gene expression to basal level (pre-starvation expression) after Pi re-application was within 2 to 6 h after Pi re-supply. This response occurs too rapidly to be under the control of a systemic signal.

Figure 3.8 illustrates the difference in *MGD3* gene expression kinetics after transfer to Pi starvation conditions, compared with the kinetics of recovery from Pi starvation. *MGD3* expression rose gradually with increased duration of Pi starvation (or

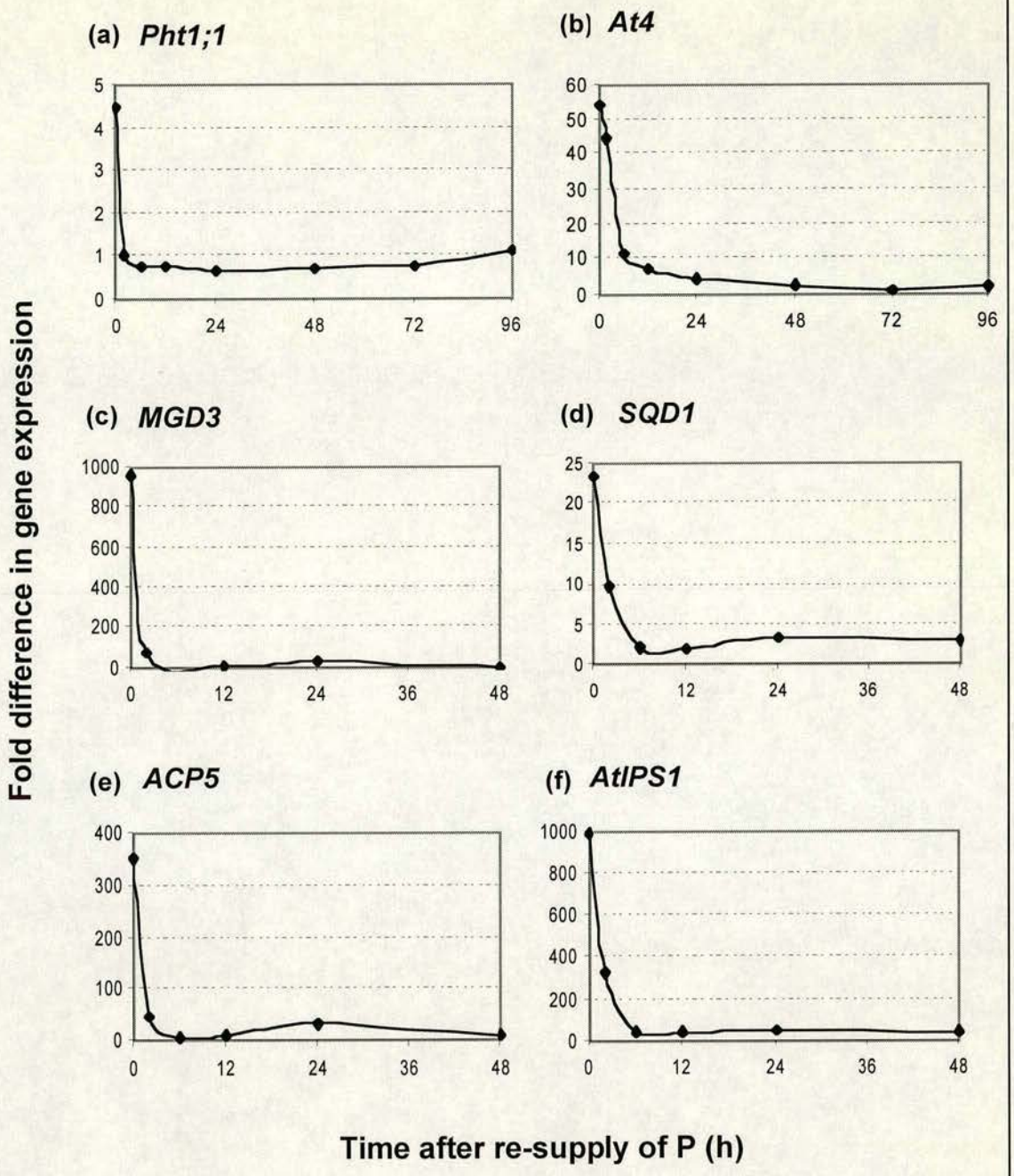


Figure 3.7: Recovery of expression of phosphate starvation response genes in *Arabidopsis* Col0 roots after re-supply of Pi to starved plants. Seedlings were pre-grown in 0.5x MS liquid media containing 500 µM Pi, transferred to no Pi media for 4 days and then to 2.5 mM Pi media. Fold difference in expression, determined by Quantitative RT-PCR, is relative to t=0 expression prior to transfer to starvation conditions and has been normalised for cDNA loading by comparing threshold cycles of amplification of the gene of interest to that of the constitutively expressed *eIF4A* gene.

decreased internal Pi stores). In contrast, recovery is almost immediate after the addition of Pi, with expression dropping from 955-fold to 71-fold within 2 h.

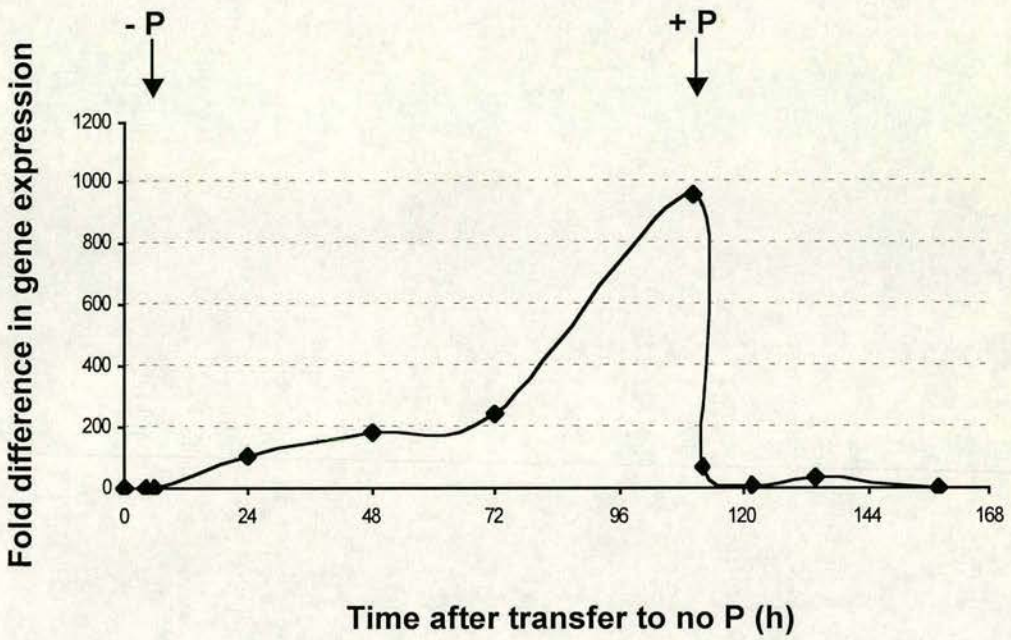


Figure 3.8: Kinetics of MGD3 expression in *Arabidopsis* Col0 roots after transfer to no Pi and then after re-supply of Pi. Seedlings were pre-grown in 0.5x MS liquid media containing 500 µM Pi, transferred to no Pi media for 4 days and then to 2.5 mM Pi media. Fold difference in expression, determined by Quantitative RT-PCR, is relative to t=0 expression prior to transfer to starvation conditions and has been normalised for cDNA loading by comparing threshold cycles of amplification of the gene of interest to that of the constitutively expressed eIF4A gene

3.3.7 *At4* expression in *At4* promoter::GFP fusion lines

To determine the localisation of *At4* expression, reporter lines were prepared. A 956bp flanking sequence of the *At4* gene representing the promoter region was transcriptionally fused to a GFP reporter gene and transformed into *Arabidopsis* Col0 plants. Homozygous seedlings from one transgenic *Arabidopsis* line harbouring the *At4* promoter::GFP construct were grown for 10 days in 2.5 mM Pi media and then

transferred to starvation conditions. The progression of GFP expression was observed. GFP expression was faintly visible after 1 day of starvation and was stronger after 2 days. Figure 3.9 shows GFP expression after 3 days of phosphate starvation. GFP expression was strongest in the vasculature and was visible only in this region under the lowest magnification (x 5 and x 10 objective). Under higher magnification (x 40 objective), GFP expression was clearly localised mainly in vascular tissues and also appeared weakly localised to vacuoles. Vascular localisation was apparent throughout the majority of the root system but was absent from the root tip and elongation zones of both primary and lateral roots. It was also absent from the hypocotyl region of the primary root and from the base of all lateral roots (as seen in Fig. 3.9a).

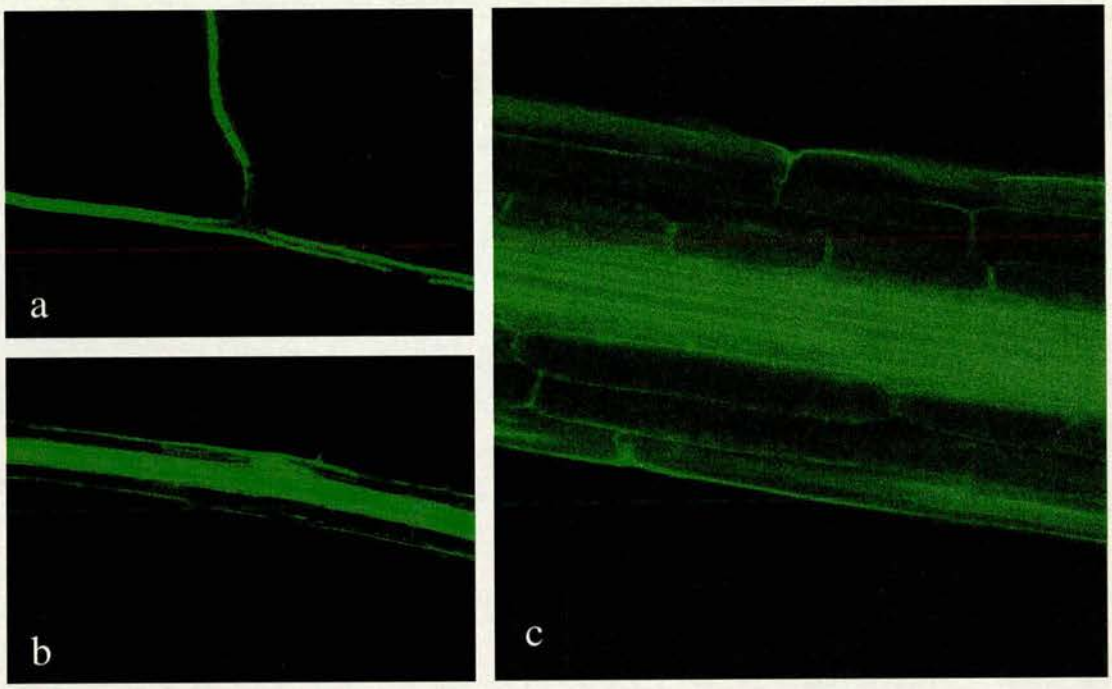


Figure 3.9: Confocal image analysis of *At4* promoter::GFP expression in response to phosphate starvation. Transgenic plants harbouring the *At4* promoter::GFP reporter gene were grown in vertical plates containing 2.5 mM Pi media for 10 days and then transferred to no Pi media for 3 days. Roots were visualised using a confocal microscope equipped with UV filter. Image (a) shows horizontal primary root with lateral root outgrowth under x 5 magnification, (b) shows primary root under x 10 magnification and (c) shows a transverse section through primary root under x 40 magnification.

3.4 Discussion

3.4.1 Molecular and physiological responses to phosphate starvation are initiated prior to visible morphological alteration

Plants initiate many responses to phosphate limitation, some of which have been characterised in detail. However, each response is often considered in relation to a singular event. Current knowledge indicates that adaptive responses to phosphate limitation involve a combination of molecular, physiological and morphological alterations. These should be examined conjointly to gain an accurate picture of the mechanisms by which plants cope with phosphate stress. The kinetic data presented here clearly illustrates that morphological alteration occurs as a secondary response to phosphate limitation. Growth rates of plants transferred from 125 μM phosphate to starvation conditions did not begin to decrease until at least 48 hours after transfer (Fig 3.4). Unlike the control seedlings that were transferred to adequate Pi conditions, growth rate did not increase during this time. Perhaps this was because adequate phosphate was not available to support enhanced growth. Nevertheless, there was no alteration in their previous pattern of growth until after 48 hours and yet there was enhanced expression of all tested genes 24 hours after transfer from the same Pi concentration (Fig. 3.5). Therefore, changes in morphology occur much later than molecular and physiological responses to phosphate starvation. Early induction of Pi-responsive genes has previously been observed in other kinetic studies of gene expression. Increases in expression were observed between 12 and 24 hours after transfer to Pi starvation conditions for *Arabidopsis Pht1;1* and *Pht1;4* Pi-transporter genes (Karthikeyan *et al.*, 2002; Mukatira *et al.*, 2001), *Arabidopsis PSR3.2* gene of unknown function (Malboobi and Lefebvre, 1997), tomato *LePT1* and *LePT2* Pi transporter genes (Liu *et al.*, 1998) and tomato TPS11 gene of unknown function. Thus far there have been no similar studies into the kinetics of root architectural responses to phosphate starvation, although Liu *et al.* (1998) report that increased root:shoot ratios do not occur until 3 to 5 days after transfer to phosphate starvation.

Steady-state experiments in which seedlings were grown under different phosphate regimes add further weight to the observation that molecular and physiological responses occur prior to morphological responses. Differences in gene expression

occurred five days after sowing yet there is no apparent morphological variation at this stage (Figures 3.2 and 3.3).

One possible explanation for these observations could be that the different signalling pathways governing all phosphate-starvation responses are activated simultaneously but it takes longer for alterations in morphology to become visually manifest. An alternative hypothesis could be that molecular and physiological changes take place primarily to scavenge for phosphate and to reduce Pi demand during initial perception of phosphate starvation. This would allow for survival during transient fluctuations in Pi availability and only after extended periods of starvation would irreversible and costly morphological alterations be initiated.

3.4.2 Phosphate availability regulates primary root length and root cortical cell length

Morphological alterations were assessed by changes in primary root length and cortical cell length. A gradual decrease in primary root length with decreasing phosphate concentration was observed across a wide range of phosphate concentrations from 1 mM no Pi, an effect that becomes more pronounced with time (fig. 3.1). Previous reports of root architectural responses reflect the outcome of the presented data (Williamson *et al.*, 2000; Linkohr *et al.*, 2002 and López-Bucio *et al.*, 2002). López-Bucio *et al.* (2002) observed a four-fold increase in primary root length between 10 and 100μM Pi and a similar maximal primary root length at 1 mM Pi. However, Linkohr *et al.* (2002) observed that 0.164 mM phosphate was the threshold concentration at which primary root growth starts to increase. In this case, primary root growth was calculated relative to shoot dry weight. This indicates that decreased root growth observed below this phosphate concentration is probably as a result of general starvation stress rather than a Pi-specific growth response.

Seedlings grown in no Pi medium, or that were transferred to no Pi medium, did not completely halt primary root growth. Instead, a very slow, but steady, rate of growth was maintained even after several days in starvation conditions (Figs 3.1 & 3.4). This ability to maintain growth is likely due to the reduced demand for Pi by the plant and because Pi starvation mechanisms activated by the plant allow for recycling and re-mobilisation of Pi reserves. The medium used for root growth experiments was

solidified with a gelling agent that contributed an extra 7.5 μM Pi (see section 2.1.3). This source of Pi was probably sufficient to allow continued slow growth in media that is considered to be starvation conditions. Primary root growth would likely be slower if this source were not available to sustain continued growth.

Alterations in primary root length in response to low Pi availability are brought about partially by changes in root cortical cell length (Fig. 3.2). The observed decreases in cortical cell length with decreasing phosphate concentration could not account entirely for the parallel decrease in primary root length. Therefore, cell division must also be altered by Pi concentration. The effect of Pi starvation on root cell elongation has previously been investigated. Williamson *et al.* (2001) found a corresponding decrease in the number of cells in the elongation zone, together with increases in lateral root length. Ma *et al.* (2003) also discovered reduced elongation rates and shorter elongation zones in primary roots of plants under phosphate limitation. Primary root elongation is therefore relinquished in favour of lateral root growth during Pi stress.

Nutrient distribution in soil can be extremely heterogeneous and phosphate is often concentrated in the upper layers of soil due to its limited mobility. My results confirm that plants reduce their primary root growth in response to phosphate deprivation. Enhanced lateral root branching is also known to occur (Williamson *et al.*, 2001; Linkohr *et al.*, 2002; and López-Bucio *et al.*, 2002). The combination of these responses allows plants to access more phosphate from the topsoil during phosphate limitation.

3.4.3 Internal phosphate status regulates phosphate starvation responses

The Pi status of a plant determines the timing and extent of phosphate starvation responses. Seedlings that were pre-grown in high Pi conditions displayed much later increases in phosphate starvation gene expression than those pre-grown at low Pi (Fig. 3.6). *Arabidopsis* plants have been shown to accumulate more internal Pi stores at higher Pi concentrations (López-Bucio *et al.*, 2002). There is also known to be significant re-translocation of stored phosphate from older leaves to younger leaves and to the root during phosphate limitation (Mimura, 1995; Schachtman *et al.*, 1998). Therefore seedlings with more internal stores will have greater Pi re-translocation, or longer periods of re-translocation, and will consequently experience starvation later than seedlings with low Pi stores. Other evidence for the regulation of global Pi responses by internal rather than external Pi levels comes from split-root experiments. Expression of

Pi-responsive genes is down-regulated in the starved half of a divided root system, the other half of which is provided with phosphate (Liu *et al.*, 1998b; Burleigh and Harrison, 1999). However, Burleigh and Harrison (1999) found no correlating change in phosphate concentration in the starved half of a *M.truncatula* split-root system. Therefore, signals other than phosphate are considered to mediate the down-regulation response. If this is the case then phosphate re-translocation may not control the timing and extent of responses reported here. However, it is probable that the signal controlling the down-regulation of Pi starvation response genes would also control the induction of phosphate starvation responses in plants possessing variable Pi-storage.

3.4.4 Early *Pht1;1* expression indicates possible local control

The consistent transient increase in *Pht1;1* expression after only 2 hours of Pi starvation (fig 3.6) was an unexpected result, given that previous kinetic expression analyses have indicated 12 to 24 hours as the earliest induction of expression for any Pi-responsive gene (Malboobi and Lefebvre, 1997; Liu *et al.*, 1998b; Dong *et al.*, 1999; Mukatira *et al.*, 2001; Karthikeyan *et al.*, 2002). However, these kinetic analyses did not include the first few hours after transfer to starvation conditions. The observed early peak of *Pht1;1* expression occurs too early to be governed by a systemic signal and suggests that *Pht1;1* may be under the control of local phosphate availability. This seems a logical conclusion considering the location of *Pht1;1* transcripts in root hair-forming trichoblast cells (Karthikeyan *et al.*, 2002; Mudge *et al.*, 2002). Density and elongation of root hairs are controlled by local phosphate availability and increases in root hair length can be observed within hours after transfer to Pi starvation (Bates and Lynch, 1996). The method by which root hairs are thought to uptake Pi is via high-affinity Pi-transporters such as those encoded by *Pht1;1*. Fast initiation or elongation of root hairs would be of no benefit to Pi acquisition if Pi transporters were not in place equally as quickly. Local control of Pi-transporter gene transcription would thus be a prudent strategy. Whether local responses are due to internal or external Pi status is unclear. Perhaps the root is somehow able to sense external Pi concentration. Alternatively, the Pi status of root epidermal cells may trigger changes in *Pht1;1* expression, as this is the only plant tissue that could potentially experience such a quick change in Pi concentration.

There are other indications that expression of the Pht1 family of high-affinity Pi-transporters may not be under the control of shoot Pi status. The expression of *Pht1;1*

and Pht1;2 is unaltered in the *pho1* and *pho2* translocation mutants (Smith *et al.*, 1997). As *pho1* has 20-fold less Pi in its leaves (Poirier *et al.*, 1991) and *pho2* has up to five-fold more Pi in its leaves, compared to wild-type levels (Delhaize and Randall, 1995), this suggests that the expression of these transporter genes responds to root, rather than shoot, Pi status,

Early induction of other newly-identified Pi-response genes has recently been reported in tomato. These genes, including MAP kinases, a putative transcription factor and a 14-3-3 protein, were also up-regulated by other nutrient deficiencies and induction occurred within 1 to 3 h in most cases (Wang *et al.*, 2002). Expression was transient, decreasing to basal level within 6 to 12 h, reminiscent of *Pht1;1* expression. Wang *et al.* (2002) speculated that these early-induced genes have an early signalling role, possibly as root-derived signals communicating external Pi status to the shoot. This is clearly not the case for *Pht1;1* as its role as a Pi-transporter is well established.

Research into ribonuclease gene expression (RNase LX) in tomato cell cultures provides an insight into what may be occurring to produce early, transient *Pht1;1* expression in *Arabidopsis* roots. Gene expression in plant cell cultures can only be affected by local signals and by local phosphate concentration. The extracellular administration of phosphate-sequestering metabolites in the presence of high Pi resulted in a peak of RNase LX expression after 2 hours, which then rapidly decreased after futher uptake of extracellular Pi. This research proved that the sensing mechanism inducing activation of RNase LX gene expression is intracellular (Köck *et al.*, 1998). It is understood that when whole plants initially encounter phosphate starvation, cytoplasmic Pi levels will be buffered by vacuolar Pi reserves. However, measurements of Pi content in cytoplasmic and vacuolar compartments of sycamore cells by ^{31}P-NMR (Roby *et al.*, 1986) and examination of Pi content in isolated barely vacuoles (Martinoia *et al.*, 1985) indicate that Pi efflux from the vacuole may be very slow. There is therefore an interval immediately after transfer to starvation conditions during which low levels of cytoplasmic Pi are not replenished by vacuolar stores. This may well be the trigger for early induction of *Pht1;1* expression and of the genes identified by Wang *et al.* (2002). The subsequent decrease in expression could thus be accounted for by the eventual buffering of cytoplasmic Pi levels by vacuolar Pi.

3.4.5 Possible local control of all phosphate starvation response genes

There is evidence from split-root experiments that some Pi-starvation response genes are under systemic control (Burleigh and Harrison, 1999; Liu *et al.*, 1998b). The delay of 12 to 24 hours after transfer to starvation conditions before increases in expression of many Pi-responsive genes are seen, may also be indicative of systemic control dependent on shoot internal levels. Kinetic experiments also found delays in expression depending on the level of stored Pi (fig 3.6). There have been some suggestions that sufficient whole plant Pi-status has a shielding effect on Pi-starvation signalling and responses (Abel *et al.*, 2002). This led us to question whether Pi responses would occur faster if a plant did not possess vacuolar Pi stores to replenish cytoplasmic levels. The re-supply of phosphate to starved plants with depleted Pi stores, created conditions in which signalling would not be shielded by Pi storage and re-cycling. It transpired from these experiments that mRNA levels of all Pi-starvation response genes recovered to basal levels within 2 to 6 hours after Pi re-supply. This exceptional speed of transcriptional de-activation probably rules out the systemic control of these genes during re-supply. Previous studies into recovery of gene expression have mainly focused on days after re-supply, rather than hours. The first time-point of analysis in these experiments tends to be 24 hours after re-supply, by which time expression is mostly recovered to basal level. This includes recovery analysis of *Arabidopsis Pht1;1*, *Pht1;2* and *Pht1;4* high affinity-Pi-transporters (Dong *et al.*, 1999; Karthikeyan *et al.*, 2002) and the tomato *LePT1* and *LePT2* high-affinity Pi-transporters (Liu *et al.*, 1998b). Timing of recovery of gene expression in these experiments could therefore have occurred at any time within the first 24 hours after re supply. One exception is the *AtACP5* Pi re-supply experiment by del Pozo *et al.* (1999), which showed complete recovery only after 4 days, a result in disagreement to the outcome obtained here.

Plants pre-grown in low phosphate environments have an earlier induction of responses to starvation conditions, as was observed in kinetic starvation experiments. If the tested molecular and physiological responses are under local control, then plants pre-grown at even lower Pi concentrations should display earlier induction of starvation responses. Furthermore, plants pre-grown in Pi concentrations too low to allow Pi storage may theoretically result in the detectable induction of molecular responses as rapidly as 2 to 6 hours after transfer to starvation conditions.

The rate of mRNA turnover must be extremely fast for transcripts to be no longer detectable after a few hours of Pi re-application. This is consistent with the identification of short-lived Pi-inducible RNase transcripts in tomato cell cultures, which were likewise turned over within 2 to 4 hours (Köck *et al.*, 1998).

3.4.6 Phosphate starvation gene expression: local and systemic control?

The results presented in this chapter indicate that phosphate starvation gene expression is controlled by local Pi concentration. In seedlings transferred to phosphate starvation conditions, only *Pht1;1* expression was found to be immediately and transiently up-regulated, probably triggered by changes in local or cytoplasmic Pi concentration. If this is the case, then it might have been expected that the expression of the other Pi-response genes should also have been triggered. However, all other Pi starvation response genes were not induced until 24 - 48 hours later (including *Pht1;1*), indicating that these genes were under the control of whole plant Pi-status and possible systemic control. In contrast, the down-regulation of all genes after Pi re-supply appeared to be controlled by local or cytoplasmic Pi levels, due to the rapidity of the response. This could suggest that the induction of Pi starvation response genes is under systemic control, whereas down-regulation of the same genes is under local control. However, the delay in the induction of Pi starvation response genes does not necessarily indicate systemic control. Local phosphate starvation signals may be delayed in plants previously grown in adequate phosphate conditions, due to the presence of vacuolar Pi stores which buffer cytoplasmic Pi levels. Perhaps only once all vacuolar reserves are exhausted would the presence of low cytoplasmic Pi levels result in the induction of local responses.

Evidence for the existence of systemic signals controlling the down-regulation of Pi-response genes has also previously been documented (Liu *et al.*, 1998; Burleigh and Harrison, 1999). This raises the possibility that both local and systemic signals exist to control phosphate starvation responses in *Arabidopsis*. The issue of local and systemic Pi starvation signals is pursued further in chapters 5 and 6 and an overview of Pi starvation signalling is presented in chapter 7 (Conclusion).

3.4.7 *At4* is expressed mainly in vascular tissue

At4 promoters strongly activated GFP reporter gene expression in the vasculature of *Arabidopsis* plants. GFP was found to be expressed throughout the vascular tissue of

primary and secondary roots but was notably absent from root tips and elongation zones. Expression was also lacking from the base of lateral roots and the hypocotyl region of the primary root. Early stages of Pi starvation resulted in weak GFP activity and longer periods of Pi starvation resulted in an intensification of GFP activity in the same locations. The *Arabidopsis AtIPS1* gene belongs to the same gene family as *At4* but clearly has a different expression pattern. Analysis of GUS activity in transgenic plants harbouring the *AtIPS1*::GUS reporter fusion revealed that GUS activity was restricted mainly to cotyledons and to the elongation zone of the root during early exposure to Pi starvation conditions. Prolonged periods of Pi starvation resulted in GUS expression throughout the whole plant (Martin *et al.*, 2000). The differences in expression pattern observed between *At4* and *AtIPS1*, particularly during early stages of Pi limitation, may reflect differing roles in the phosphate starvation response.

CHAPTER 4: THE EFFECT OF CARBON AVAILABILITY ON THE *ARABIDOPSIS* PHOSPHATE STARVATION RESPONSE

4.1 Summary

The interaction between phosphate, photosynthesis and carbon metabolism is important and extremely complex. A balance of carbon and phosphate pools is likely necessary for normal plant growth and metabolism and previous research has found that both growth and gene expression can be affected by perturbations of the C:P ratio. Here, increasing the C:P ratio by supplying exogenous sucrose to *Arabidopsis* plants was found to enhance phosphate starvation responses at the level of growth and gene expression. Increased C availability may exaggerate Pi starvation by speeding up metabolism or by decreasing cellular phosphate pools.

4.2 Introduction

Phosphate is an essential component of plant metabolism and many metabolic pathways require phosphate, pyrophosphate, ATP, ADP and AMP to proceed. The importance of phosphate to metabolism is such that alternate metabolic pathways exist to circumvent Pi-requiring steps to allow metabolism to continue during phosphate limitation (see section 1.9). Carbon is essentially the critical element of metabolism and sucrose is the initial substrate for glycolysis. It therefore seems logical that metabolism, which relies both on carbon and phosphate, may be affected by the relative ratios of these nutrients. Plants grown in low phosphate conditions tend to accumulate carbohydrate in roots and shoots. This suggests that carbohydrate utilisation is lower in Pi-deficient plants and illustrates the intricate relationship between the two nutrients (Paul and Stitt, 1993).

Carbon is fixed from CO_2 via the process of photosynthesis, whereas phosphate is acquired from the soil. Therefore, supplementing plant growth media with sucrose provides an unnatural environment for a plant to inhabit. Nevertheless, uptake of exogenous sucrose by plants does occur and this can be exploited to determine the effect that the C:P ratio has on a plant. Previous studies involving the application of exogenous sucrose to plants, resulted in increased growth and altered root-to-shoot ratios of plants grown in adequate Pi conditions, together with corresponding increases in respiratory

enzyme activity. However, growth was found to be unaffected in Pi-deficient plants supplied with exogenous sucrose (Paul and Stitt, 1993). Evidence that carbon: phosphate ratios control the expression of some genes was provided by Sadka *et al.* (1994) who discovered that soybean *VspA* and *VspB* sugar-inducible genes are also activated during phosphate starvation. It is therefore evident that the ratio of these two nutrients may have profound effects on plant responses to nutritional stress and that the balance of nutrients may be critical to plant metabolism, growth and gene expression. However, information on this subject is lacking and more research needs to be conducted to further understand the relationship.

The ratio of carbon to another soil-acquired macro-nutrient, nitrogen, has recently been studied and may provide some clues. Growth and gene expression are both affected by the ratio of these two nutrients and enhanced nitrate starvation responses have been shown to occur in the presence of high exogenous sucrose. Lateral root initiation was repressed and anthocyanin accumulation increased, whereas photosynthetic gene expression and chlorophyll content decreased further under these conditions (Malamy and Ryan, 2001; Martin *et al.*, 2002). The extent of the nitrate starvation response is therefore dependent not solely on nitrate availability but on the relative ratio of nitrate to carbohydrate availability.

Thus, the aim of the experiments discussed in this chapter is to determine the effect of carbon availability on plant phosphate starvation responses by providing an exogenous sucrose source and observing Pi-starvation responses. Primary root length and primary root cortical cell length will be assessed to determine growth responses. Molecular responses will be represented by the analysis of *Pht1;1*, *At4*, *AtIPS1* and *AtACP5* gene expression. Indicators of physiological responses will include *MGD3*, *SQD1* and *PEPC* gene expression.

4.3 Results

4.3.1 The effect of carbon availability on *Arabidopsis* growth responses to phosphate starvation

To determine whether carbon availability affected root growth responses to phosphate, *Arabidopsis* seedlings were grown in vertical plates containing media with different Pi and C concentrations. Primary root length was recorded daily.

There was little difference in primary root length between all conditions after 5 days (Fig 4.1a). However, by day 6, seedlings grown in no Pi or 125 μM Pi in the presence of 3% sucrose had shorter primary roots than seedlings grown in 0.3% and 2% sucrose (between which there was little difference). Primary root length of seedlings grown in 3% sucrose remained shorter throughout the duration of the experiment. Conversely, seedlings grown in the highest Pi condition of 2.5 mM Pi had longer roots in 2% or 3% sucrose than those grown in 0.3% sucrose.

Figure 4.1b illustrates the corresponding rate of primary root growth of the same seedlings. By day 6, seedlings grown in no Pi had much slower root growth at 3% sucrose (1.5 mm/ day) compared to those grown at 2% sucrose (3.4 mm/ day) and at 0.3% sucrose (4.4 mm/ day). Root growth rate continued to decrease over time in no Pi at all sucrose concentrations. Declining primary root growth rates ceased by day 9 for seedlings grown at 3% sucrose but growth continued at a very slow, steady rate of 0.2 mm/ day. It should be noted that this value is representative of mean growth rate and that many roots had stopped growing after day 9 in this growth condition. At the lower sucrose concentrations, root growth rate continued to decrease until day 11, at which point it had declined to 0.6 mm/ day and 0.45 mm/ day at 0.3% and 2% sucrose, respectively. Seedlings grown in 125 μM Pi had a similar growth response pattern. By day 6, primary root growth rate at 3% sucrose was much slower (1.7 mm/ day) than at 2% and 0.3% sucrose (4.21 and 3.35 mm/ day, respectively). Root growth rate at 3% sucrose increased slightly until day 9 and then decreased, whilst growth rate at 0.3% and 2% sucrose gradually decreased over time. By day 11, root growth rates at all three sucrose concentrations were similar (~1.5 mm/ day). The opposite response was observed for seedlings grown in 2.5 mM Pi. Root growth rate by day 6 was fastest in the highest sucrose concentrations (5.8 mm/ day at 2% sucrose and 6.0 mm/ day at 3%

sucrose). These rates remained steady over time. However, at 0.3% sucrose, root growth was slower by day 6 (5.1 mm/ day) and gradually decreased over time to 4.0 mm/ day by day 11.

Therefore, increasing C availability resulted in slower primary root growth of Pi-limited plants but enhanced primary root growth in plants grown in high Pi.

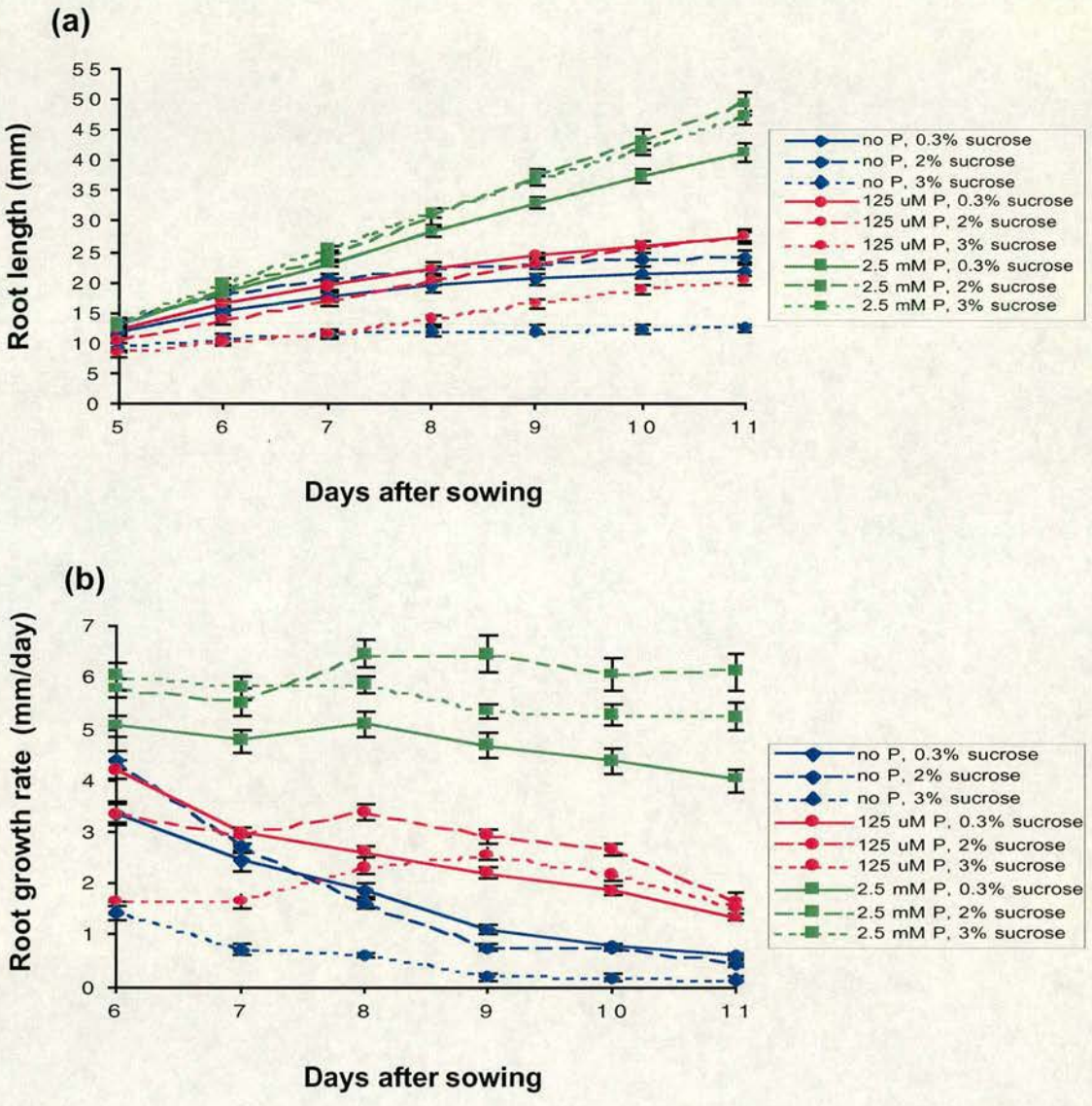


Figure 4.1: The effect of varying phosphate and sucrose concentration on a) primary root length and b) primary root growth (mm/ day). *Arabidopsis* Col0 seeds were sown on vertical plates containing 0.5x MS with no Pi, low Pi (125 M) and high Pi (2.5 mM), at different sucrose concentrations. Root length was marked after 5 days and every subsequent 24 h. Values represent means of 15-25 seedlings (+/- SE). Data represent one of three independent repeat experiments.

To determine whether root cortical cell length was affected by the C:P ratio, GFP Q8 PIP2A seedlings were grown under different phosphate and sucrose concentrations. Primary root length was compared with the length of cortical cells in the same roots. Primary root length was similar in all phosphate and sucrose concentrations 5 days after sowing (see figure 4.1a), as was root cortical cell length (Figure 4.2). By day 11, primary roots of seedlings grown in no Pi or 125 μM Pi, with 3% sucrose, were shorter than at 2% or 0.3% sucrose. Conversely, seedlings grown in 2.5 mM Pi had shorter primary roots in 0.3% sucrose than in 2% and 3% sucrose (Fig 4.3a). However, cortical cell length was not correspondingly affected by sucrose concentration (Fig. 4.3b)

Cortical cell length has been shown to be altered by Pi concentration. Increases in root length in response to phosphate are therefore probably due to an increase in root cortical cell length (see section 3.3.1). However, the decrease in root length observed under higher sucrose concentrations was not accompanied by a decrease in cortical cell length, therefore a change in root length caused by sucrose addition can only be attributed to a change in root cell division.

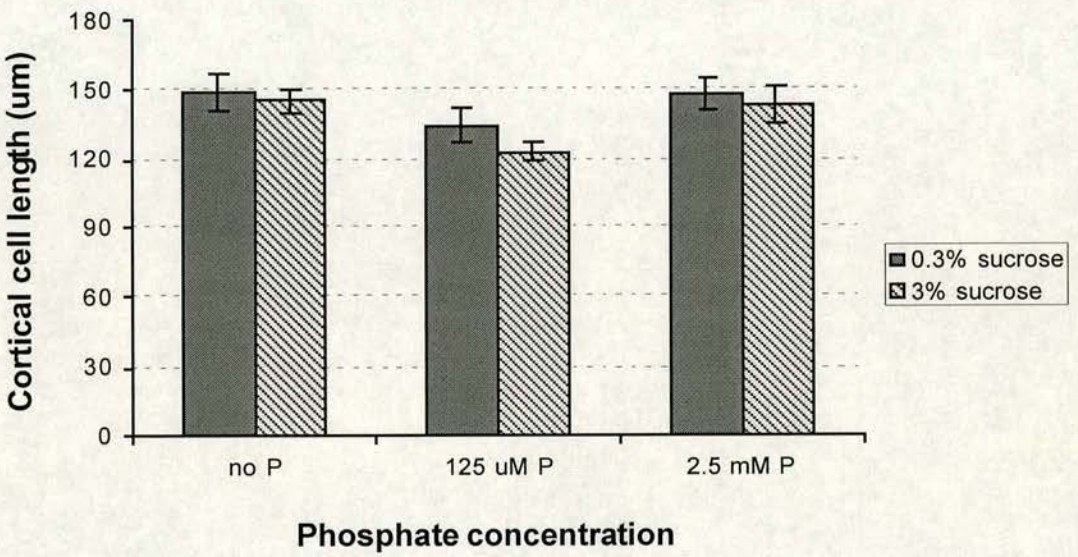


Figure 4.2: Root cortical cell length of *Arabidopsis* Q8 PIP2A seedlings 5 days after sowing in vertical plates on 0.5x MS media containing no Pi, low Pi or high Pi at 2 different sucrose concentrations. Data represents mean cell length (+/- SE) pooled from 6 different root samples. From each root, 15 cortical cells located approximately 0.5cm above the elongation zone, were measured. Data represent one of two independent repeat experiments.

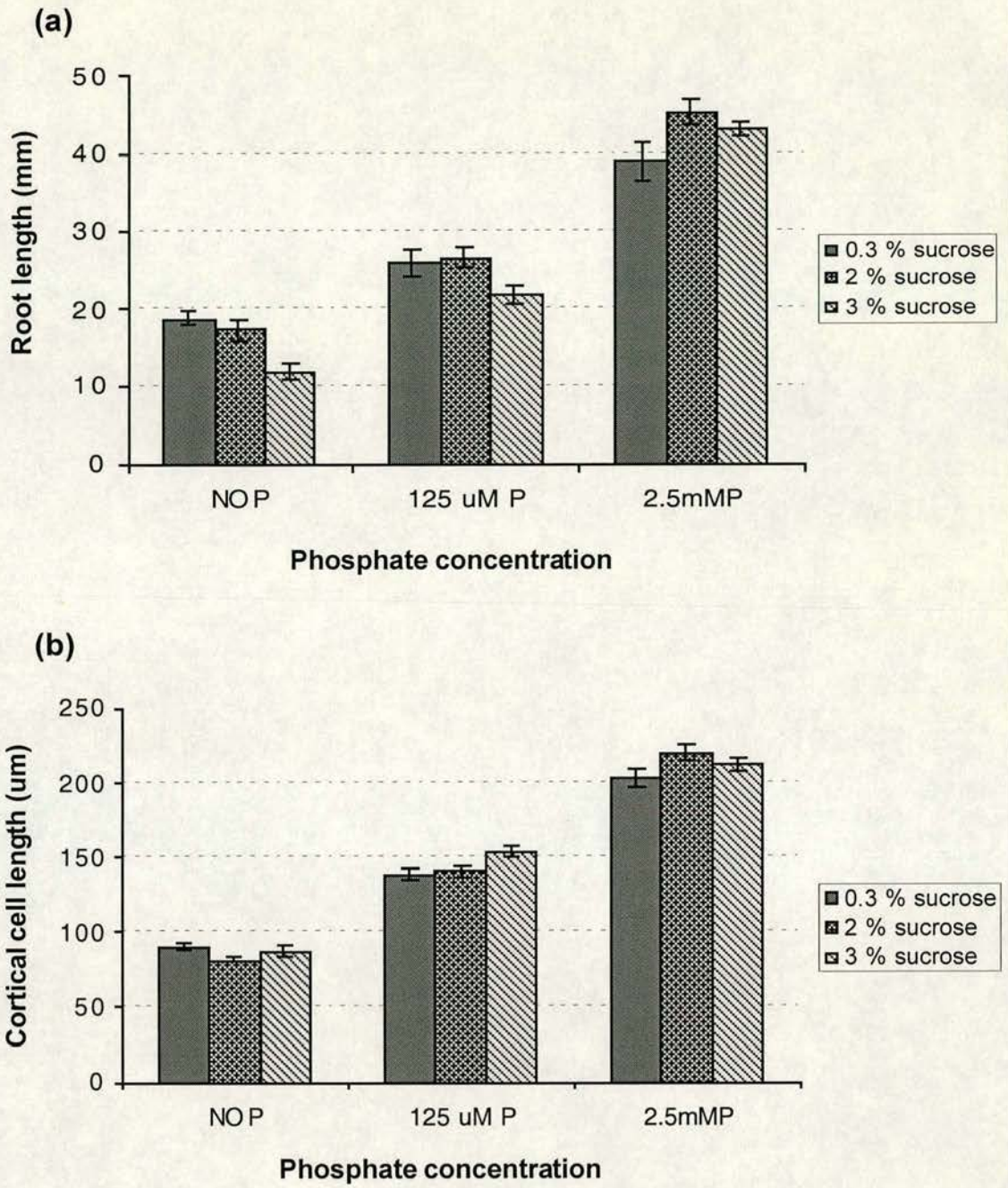


Figure 4.3: The effect of phosphate and sucrose concentration on a) primary root length and b) root cortical cell length of *Arabidopsis* Q8 PIP2A seedlings. Seedlings were germinated and grown in vertical plates on 0.5x MS media containing no Pi, low Pi or high Pi at 3 different sucrose concentrations for 11 days. Root length data represents mean primary root length (+/- SE) of 15-25 seedlings. Cortical cell length data represents mean cell length (+/- SE) pooled from 6 different root samples. From each root, 15 cortical cells located approximately 0.5 cm above the elongation zone, were measured. Data represent one of three independent repeat experiments.

4.3.2 The effect of carbon availability on *Arabidopsis* molecular and physiological responses to phosphate starvation

The expression of Pi starvation-inducible genes was analysed to determine whether a difference in C availability had an effect on molecular and physiological responses to Pi starvation. Seedlings were grown on different Pi and sucrose media, root tissue was sampled, and phosphate-starvation inducible gene expression was determined by Quantitative RT-PCR analysis. Samples were taken, 5, 7 and 11 days after sowing, but data from day 11 was inconclusive (seedlings grown in no Pi conditions were close to death at that time and Pi drainage to the bottom of vertical plates over time reduced the homogenous distribution of Pi within the media).

After 5 days under different Pi and sucrose regimes there were considerable differences in gene expression between seedlings grown under different phosphate conditions. All Pi starvation-inducible genes tested showed higher expression in lower Pi conditions as expected. Furthermore, even higher levels of expression were found in seedlings grown in the same phosphate condition but under higher sucrose concentrations (Fig. 4.4). After 5 days in no Pi or 125 μM Pi, expression of all genes at 3% sucrose was at least double the level of expression found at 0.3% sucrose. However, after 7 days in no Pi media, gene expression was actually lower at 3% sucrose than at 0.3% sucrose (with the exception of *SQD1*). This was unexpected but was likely due to the observation that seedlings grown in these conditions were extremely stressed and close to death. In contrast, seedlings grown at 125 μM Pi for 7 days had higher *At4*, *AtIPS1* and *SQD1* expression at 3% sucrose than at 0.3% sucrose. In addition, although the expression of these genes was generally much lower at 2.5 mM Pi, seedlings grown at this phosphate concentration with 3% sucrose displayed higher levels of gene expression than seedlings grown at 0.3% sucrose. This was observed after both 5 and 7 days.

PEPC expression (Fig 4.4b) did not vary much with phosphate concentration but there was a considerable increase in expression at all Pi concentrations, in roots of seedlings grown in 3% sucrose compared with 0.3% sucrose.

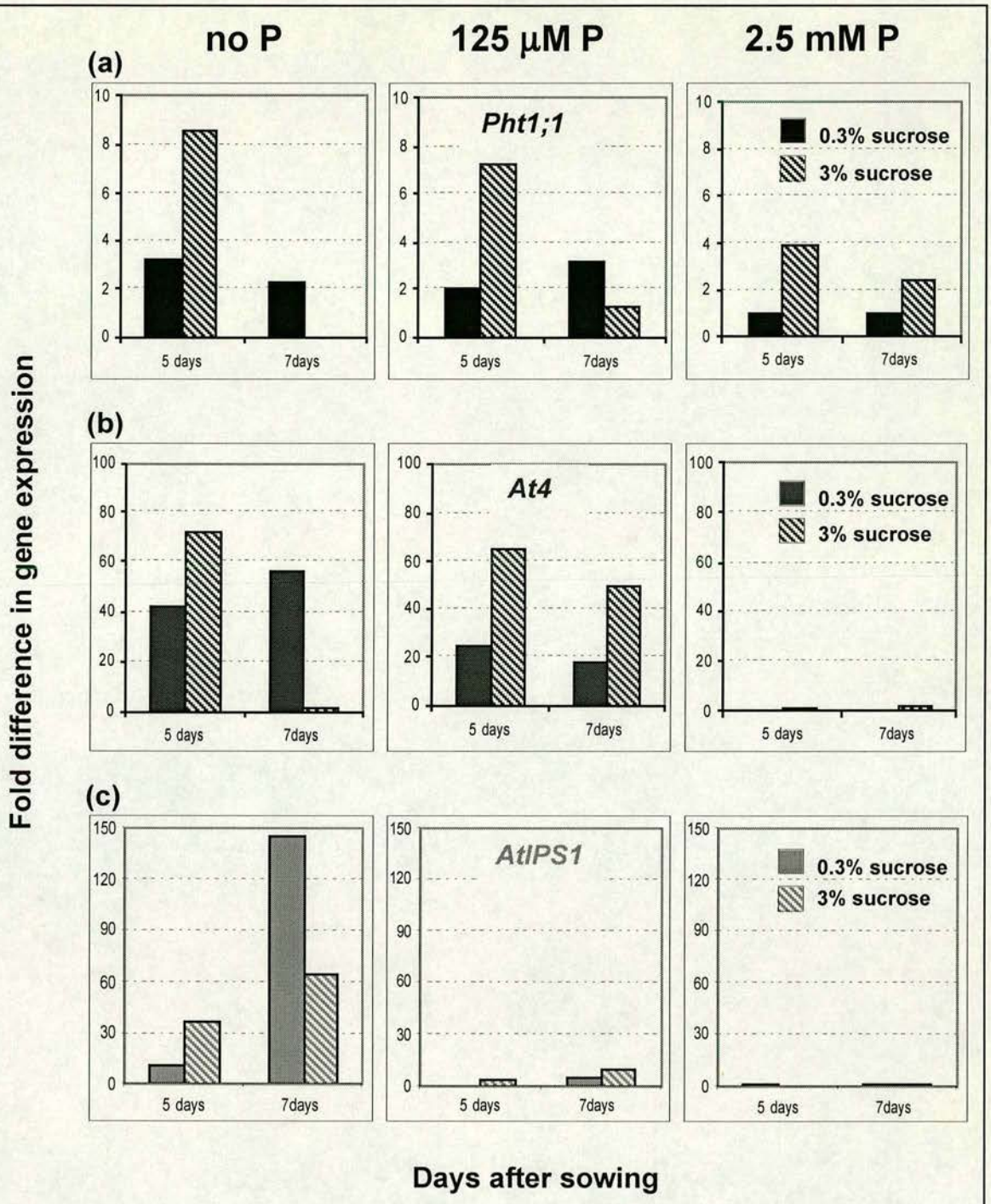


Figure 4.4: The effect of sucrose concentration on the expression of phosphate starvation response genes a) *Pht1;1*, b) *At4* and c) *AtPS1* in roots of *Arabidopsis* Col0 seedlings. Root tissue was sampled 5 and 7 days after seeds were sown on 0.5x MS vertical plates containing no Pi, low Pi (125 µM) or high Pi (2.5 mM) and either 0.3% or 3% sucrose. Fold difference in expression, determined by Quantitative RT-PCR, is relative to expression of 2.5 mM P (0.3% sucrose) 5 day sample and has been normalised for cDNA loading by comparing threshold cycles of amplification of the gene of interest to that of the constitutively expressed *eIF4A* gene. Data represent one of three independent repeat experiments.

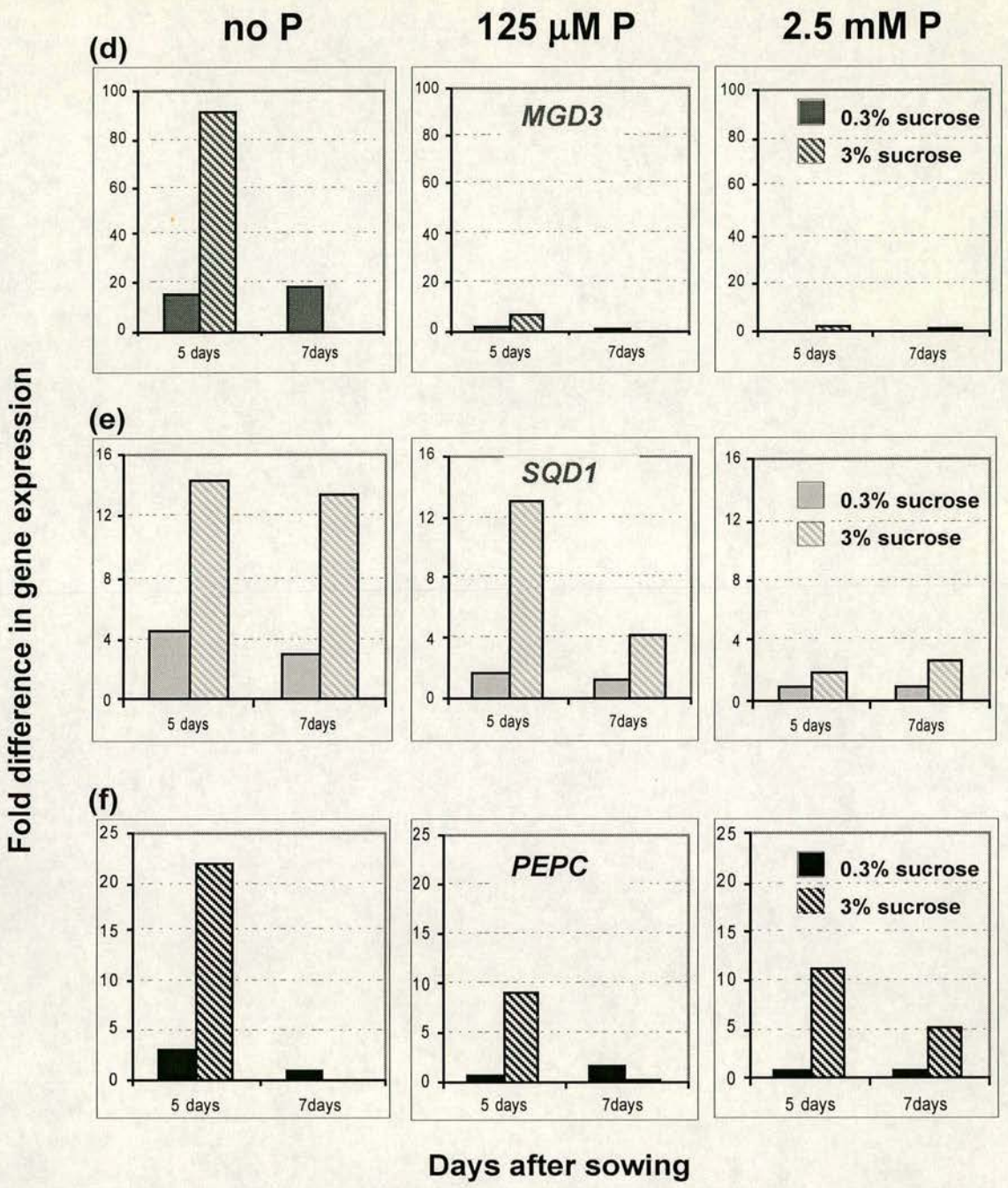


Figure 4.4 (continued): The effect of sucrose concentration on the expression of phosphate starvation response genes d) *MGD3*, e) *SQD1* and f) *PEPC* in roots of *Arabidopsis* Col0 seedlings. Root tissue was sampled 5 and 7 days after seeds were sown on 0.5x MS vertical plates containing no Pi, low Pi (125 µM) or high Pi (2.5 mM) and either 0.3% or 3% sucrose. Fold difference in expression, determined by Quantitative RT-PCR, is relative to expression of 2.5 mM (0.3% sucrose) 5 day sample and has been normalised for cDNA loading by comparing threshold cycles of amplification of the gene of interest to that of the constitutively expressed *eIF4A* gene. Data represent one of three independent repeat experiments.

4.4 Discussion

4.4.1 The C:P ratio alters root growth responses to Pi availability

Primary root length serves as an indicator of whole plant growth responses to phosphate availability. Plants grown in low Pi conditions have previously been found to have a re-distribution of root growth from the primary root to the lateral roots (Williamson et. al., 2001). It was shown in section 3.31 that primary root growth decreases with decreasing Pi concentration. Providing high exogenous sucrose (3% w/v) to Pi-limited plants resulted in even shorter primary roots. Primary root growth of many seedlings grown in starvation conditions completely ceased after several days (Fig. 4.1). Clearly, the Pi contamination (7.5 μM) contributed by the gelling agent is no longer sufficient to sustain a slow, steady growth rate if high levels of sucrose are also provided. Therefore, the consequence of increasing the internal C:P ratio of an already Pi-limited plant is an enhanced Pi-starvation growth response. This at first appears contradictory to the results obtained by Paul and Stitt (1993) and Williamson *et al.* (2001) both of whom recorded no alterations in root system architecture in low Pi plants supplied with exogenous sucrose. However, experiments conducted with tobacco plants (Paul and Stitt, 1993) are not directly comparable to experiments conducted using *Arabidopsis*. Also, much lower levels of exogenous sucrose (1% w/v) were applied by Williamson *et al.* (2001). This is consistent with my findings that sucrose concentrations of 2% and below had no effect on primary root growth. It therefore appears that only extremely high C:P ratios can affect root growth responses of low Pi seedlings. Conversely, plants grown in high Pi conditions had increased primary root growth under higher sucrose conditions (2% and 3% w/v). This parallels previously recorded observations of increased root fresh weight in tobacco seedlings provided with exogenous sucrose under adequate Pi conditions (Paul and Stitt, 1993). Primary root growth is therefore limited in plants growing in more than adequate Pi conditions, if C availability is low. Evidently, a certain balance of C:P is required for optimum growth.

These results indicate that primary root growth is not only regulated by Pi availability but also by the C:P ratio. Alterations in primary root length in response to higher C:P were not accompanied by changes in cortical cell length and are thus due to alterations in cell division, whereas primary root length alterations in response to Pi are

at least partly due to changes in cell expansion (see section 3.3.1). Therefore, signalling pathways controlling root length in response to C:P must be different to signalling pathways controlling root responses solely to Pi.

4.4.2 The C:P ratio alters molecular and physiological responses to Pi availability

Evidence for the enhanced expression of Pi-starvation specific genes in the presence of high carbon availability is presented here for the first time. All genes tested displayed this response. These represent a range of responses to Pi starvation, including genes that encode high-affinity Pi-transporters, an acid phosphatase and genes of unknown function. Genes involved in physiological responses to Pi starvation were also expressed to higher levels under high sucrose concentrations. For example, genes involved in the biosynthesis of galactolipds and sulfolipids, which replace membrane phospholipids during Pi stress, were induced to higher levels. Altering C:P ratios may also have implications for metabolism. *PEPC* has a specific metabolic role and the increase in *PEPC* mRNA transcripts under higher sucrose conditions is indicative of metabolic alteration. *PEPC* is necessary for organic acid synthesis and is responsible for increased organic acid production under Pi starvation (Johnson *et al.*, 1996a, 1996b; Neumann and Romheld., 1999; Aono *et al.*, 2001). The activity of *PEPC* has previously been shown to increase during Pi starvation (Duff *et al.*, 1989), although mRNA levels did not seem to vary much with Pi concentration in the experiments conducted here. However, the increase in *PEPC* gene expression observed with sucrose addition may be a consequence of Pi depletion brought about by high C:P ratios. Further tests would be required to substantiate this.

Interestingly, high sucrose concentrations resulted in slightly enhanced gene expression even at the highest Pi concentration. This was unexpected considering that the same condition resulted in increased primary root growth. This indicates that when both C and Pi are over-supplied (i.e. are present in more than adequate amounts) the plant perceives a minor Pi stress and Pi-starvation genes are induced accordingly. Yet, at the same time, primary root growth continues at optimum rate. Perhaps this is indicative of their different regulation. The sensing mechanism for activation of a Pi-inducible RNase in tomato cell cultures was found to be intracellular (Köck *et al.*, 1997) and it stands to reason that many other Pi-inducible genes are regulated by intracellular Pi concentration. On the other hand, root system architecture is thought to depend primarily

on external Pi levels (López-Bucio *et al.*, 2002). Thus, the differences in internal and external Pi concentration may account for the observed disparity in responses.

4.4.3 Possible causes of enhanced Pi starvation responses under high C availability

Increasing C availability at low Pi concentrations resulted in enhanced Pi starvation responses at the level of growth and at the level of gene expression. One possible reason for this effect is that increased cellular C compels metabolism to occur at a faster rate. Alternative metabolic pathways that are activated under Pi-stress still require some Pi to proceed. Therefore, faster metabolism in the presence of high C will likely exhaust Pi reserves quicker. The application of sucrose to Pi-limited tobacco seedlings was found to be associated with a considerable increase in respiratory enzyme activity and also sucrose synthase activity (Paul and Stitt, 1993). This finding points towards increased metabolism in the presence of high C that may, in turn, reduce Pi reserves faster and enhance Pi starvation responses.

Decreases in cellular Pi levels by increased C status may also be brought about by free Pi becoming bound up in sugar phosphate complexes. This is supported by evidence presented by Krapp *et al.* (1993) that the addition of glucose to *Chenopodium* cell cultures led to a sharp rise in intracellular hexose-phosphates. The relationship between carbon availability and cytoplasmic Pi was further illustrated by Rebeille *et al.* (1985) in experiments with sycamore cell suspension cultures. Cellular organic phosphate molecules were seen to degrade during sucrose starvation, resulting in increased cytoplasmic Pi levels. As internal inorganic Pi status is considered to be the trigger for induction of phosphate starvation responses, then reduced inorganic Pi levels brought about by sucrose addition may account for the observed enhanced Pi-starvation responses.

4.4.4 Maintenance of the C:P ratio

Nutritional conditions of high C availability have been shown to result in an enhanced Pi-starvation response. However, the exogenous sucrose source provided in these experiments would not be available in a plant's natural environment. Internal C:P ratios are tightly regulated by plants in the wild. The presence of sugars has in the past been shown to repress the expression of genes associated with photosynthesis (Koch, 1996).

This self-regulation should avoid the accumulation of high levels of assimilated carbon. Furthermore, Pi starvation diminishes plant demand for carbon assimilates and this results in reduced sucrose synthesis. Pi recycling to the chloroplast also becomes restricted, which in turn limits RuBP regeneration and ATP synthesis, and thus reduces the rate of photosynthesis (Pieters *et al.*, 2000). These mechanisms to reduce C assimilation under high C, or low Pi, status may well exist to counter the potentially harmful effects of exaggerated Pi starvation that are imposed by elevated internal C status.

CHAPTER 5: SYSTEMIC SIGNALLING IN *ARABIDOPSIS* DURING PHOSPHATE STARVATION

5.1 Summary

The existence of long-range, or systemic, signals regulating Pi starvation response genes has previously been observed in plant species other than *Arabidopsis*. Here, split-root experiments provide evidence for the down-regulation of the Pi starvation response genes, *At4* and *Pht1;1*, in *Arabidopsis*. Total down-regulation of *At4* and *Pht1;1* expression in roots took several days to occur, although some down-regulation was evident after only 3 days. Down-regulation of gene expression occurred in both root and shoot tissues and kinetic studies revealed earlier down-regulation in shoot tissues. The magnitude of down-regulation was also found to be dependent on internal Pi status. Potential candidates as signals include inorganic and organic Pi and extensive Pi assays are required to shed more light on their possible role in systemic down-regulation of Pi-responsive genes.

5.2 Introduction

Some responses to phosphate starvation are known to be under the control of local phosphate availability. Root hair density and elongation are under local control (see section 1.10.3). It also appears that down-regulation of Pi-starvation genes after re-supply of phosphate is under the control of local Pi availability (see chapter 3). Yet there is also evidence from split-root experiments conducted with the model legume, *Medicago truncatula* (Burleigh and Harrison, 1999) and with tomato, *Lycopersicon esculentum* (Liu *et al.*, 1998a), that systemic signals act to down-regulate Pi starvation response genes. Therefore, the regulation of some Pi starvation responses is dependent on the whole plant internal Pi status (see section 1.10.4). Coordinated regulation of Pi-starvation response genes at the level of the whole plant should allow the necessary uptake of Pi to satisfy requirements, without expending energy on starvation responses. It may also serve to avoid the accumulation of toxic levels of Pi.

As yet, there have been no reports of systemic signalling in response to phosphate status in *Arabidopsis*. However, long-range signals are known to play a role in plant responses to other nutrients. Recent split-root studies have confirmed the

existence of shoot-derived systemic signals involved in the regulation of nitrate transporters in *Arabidopsis* (Gansel *et al.*, 2001). It seems likely that long-range signals may also regulate *Arabidopsis* responses to Pi starvation.

The aim of this chapter is to determine the existence of systemic signals regulating *Arabidopsis* Pi starvation responses. This will be conducted using split-root techniques. Previous research into long-distance signalling has involved the long-term growth of plants with the root system divided into two different nutrient conditions. Gene expression analyses in these studies were conducted on root tissue collected from only one time-point. Therefore, the kinetics of down-regulation of Pi starvation response gene expression are as yet unknown. Experiments conducted here will involve younger seedlings and samples will be taken at regular intervals after transfer to split-root conditions to determine the time-scale of down-regulation. The expression of two Pi starvation response genes will be recorded: *At4*, a gene of unknown function and an *Arabidopsis* homologue of the *M.truncatula Mt4* gene previously shown to be down-regulated by a systemic signal; and *Pht1;1*, an *Arabidopsis* high-affinity Pi-transporter. Observing two different Pi-starvation response genes should provide an indication as to whether the regulation of Pi-inducible genes by long-range signals is a general response to internal Pi status.

5.3 Results

5.3.1 Systemic down-regulation of phosphate starvation responses

Arabidopsis seedlings were subjected to split-root treatment to determine whether Pi-starvation response genes are under the control of a systemic signal. Seedlings were pre-grown in low phosphate (125 μM Pi) to limit the amount of Pi-storage, before being transferred to three-compartment plates. The root system was equally divided into two separate compartments containing variable phosphate concentration. Seedlings were grown for 5 days and Quantitative RT-PCR analysis was performed on sampled root tissue. Fold difference in gene expression is relative to expression in one side of the root system of a high Pi control plant (2.5 mM).

At4 expression was high in both halves of a divided root system belonging to seedlings grown in control plates containing no Pi media in both root compartments (Fig. 5.1a). Control seedlings grown with 125 μM Pi in both root compartments had very low *At4* expression in both halves of the root system. Expression was slightly lower in the roots of control seedlings with high Pi (2.5 mM) media in both root compartments. Other seedlings were provided with different Pi concentrations in each root compartment. *At4* expression was almost completely down-regulated in the half of the root system experiencing no Pi conditions, when the other half of the root system was provided with 2.5 mM Pi. There was also down-regulation of *At4* expression in the starved half of a split-root system when the other half was in lower Pi media (125μM Pi), although down-regulation was weaker. *At4* is therefore systemically down-regulated, with the magnitude of down-regulation dependent on Pi availability.

A similar pattern of expression was also recorded for *Pht1;1* (fig 5.1b). Expression in the roots of control seedlings was high in the starved controls, low in the 125 μM Pi controls and slightly lower in the high Pi (2.5 mM Pi) controls. Systemic down-regulation of *Pht1;1* was observed in the starved half of a divided root system when the other half of the root system was provided with phosphate. However, expression levels were more variable. The magnitude of down-regulation was not evidently dependent on Pi availability. Nevertheless, *Pht1;1* expression was consistently found to be systemically down-regulated in repeat experiments.

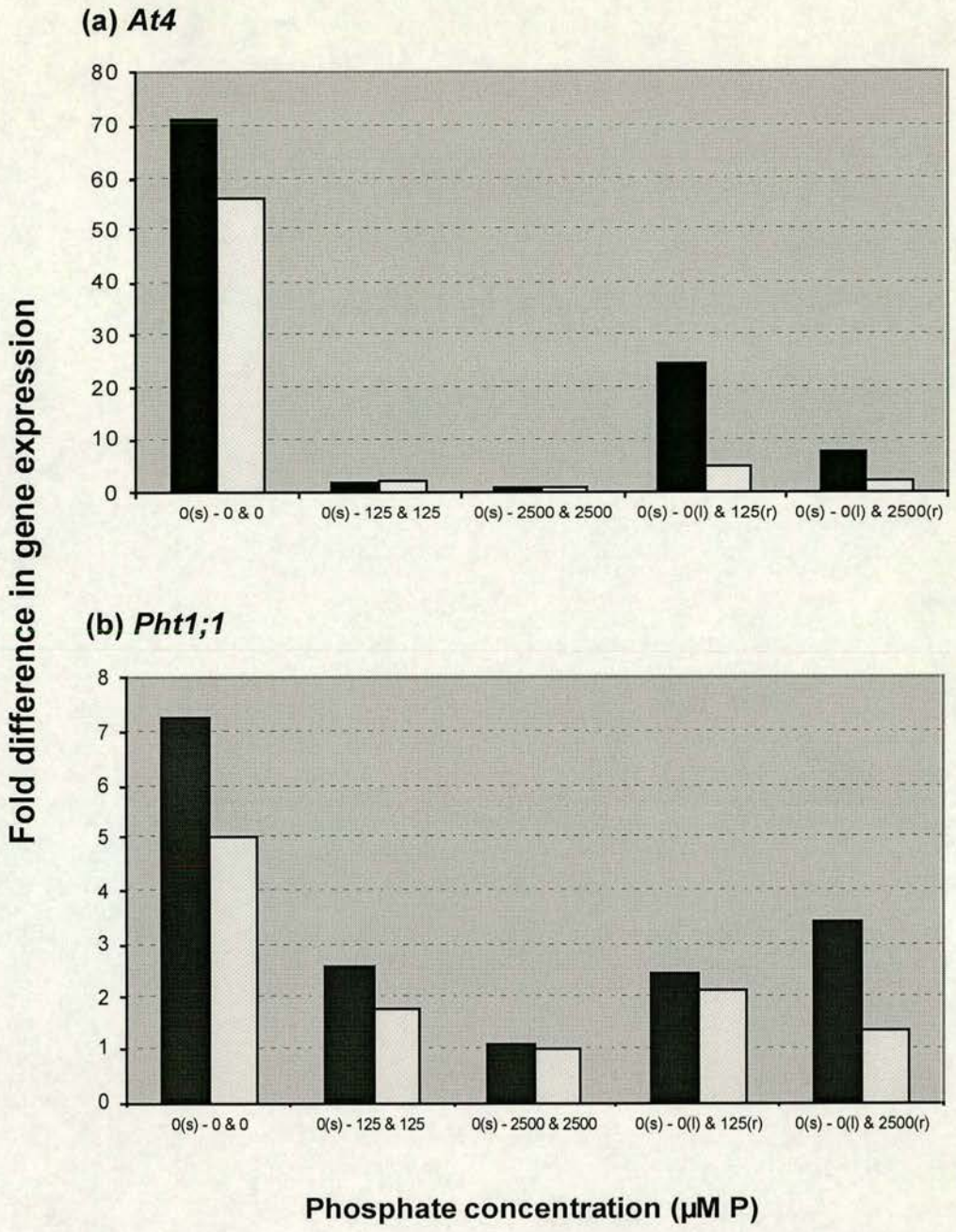


Figure 5.1: Split-root expression of a)*At4* and b)*Pht1;1* in Col 0 (wt) seedlings pre-grown in 125 µM Pi media and transferred to 3-compartment plates for 5 days. Shoots (s) were placed on solid no Pi media. Roots were divided into 2 compartments containing: no Pi in both sides (first 2 bars); low Pi in both sides (second 2 bars); high Pi in both sides (third 2 bars); no Pi in the left side and low Pi on the right side (fourth 2 bars); and no Pi on the left side and high Pi on the right side (last 2 bars). Fold difference in expression, determined by Quantitative RT-PCR, is relative to root expression of a high Pi control seedling and has been normalised for cDNA loading by comparing threshold cycles of amplification of the gene of interest to that of the constitutively expressed *eIF4A* gene. Data represent one of three independent repeat experiments.

5.3.2 Kinetics of systemic down-regulation of phosphate starvation response genes

In section 5.3.1, down-regulation of Pi-starvation response genes was observed after 5 days in split-root conditions. To investigate the kinetics of systemic down-regulation, seedlings were pre-grown in low phosphate (125 μM Pi) to limit the amount of Pi-storage and were then transferred to three-compartment plates. The root system was equally divided into two separate compartments containing variable phosphate concentration and grown for 3, 6 or 9 days. Quantitative RT-PCR analysis was performed on root and shoot tissue. Fold difference in gene expression is relative to expression in one side of the root system of a high Pi control plant (2.5 mM).

Figure 5.2a shows *At4* expression in root tissue 3, 6 and 9 days after seedlings were transferred to split-root conditions. Starved control seedlings with both halves of the divided root system grown in no Pi media, had high *At4* expression in both root halves and expression levels increased with time. High Pi control seedlings with both split-root halves in 2.5 mM Pi media, had very low *At4* expression throughout the duration of the experiment. Seedlings grown with one half of the root system in no Pi media and the other half in 2.5 mM Pi media had very low *At4* expression in the high Pi half on all days tested. In the starved half of the divided root system, gene expression was relatively high after 3 days, although it was slightly lower than in the roots of the starved controls. By day 6, gene expression in the starved half of the root system was very low and was similar to expression in the roots of high Pi control seedlings. The same result was observed for day 9. Therefore, there was some systemic down-regulation of *At4* expression after 3 days. Down-regulation was complete by day 6 and down-regulation was maintained up to 9 days after transfer.

A similar kinetic response was observed for *Pht1;1* expression (Fig. 5.2b). There was some systemic down-regulation after 3 days and complete down-regulation had occurred by day 9. However, *Pht1;1* expression was less clear than *At4* split-root expression and it seemed to vary quite considerably (as can be observed in the starved control roots on day 6). In spite of this, repeat experiments did consistently reveal systemic down-regulation of *Pht1;1* expression with similar kinetics to that of *At4* systemic down-regulation (data not shown).

At4 expression in the shoots of split-root seedlings can be seen in figure 5.3. It should be noted that expression levels are much lower compared to root expression. This

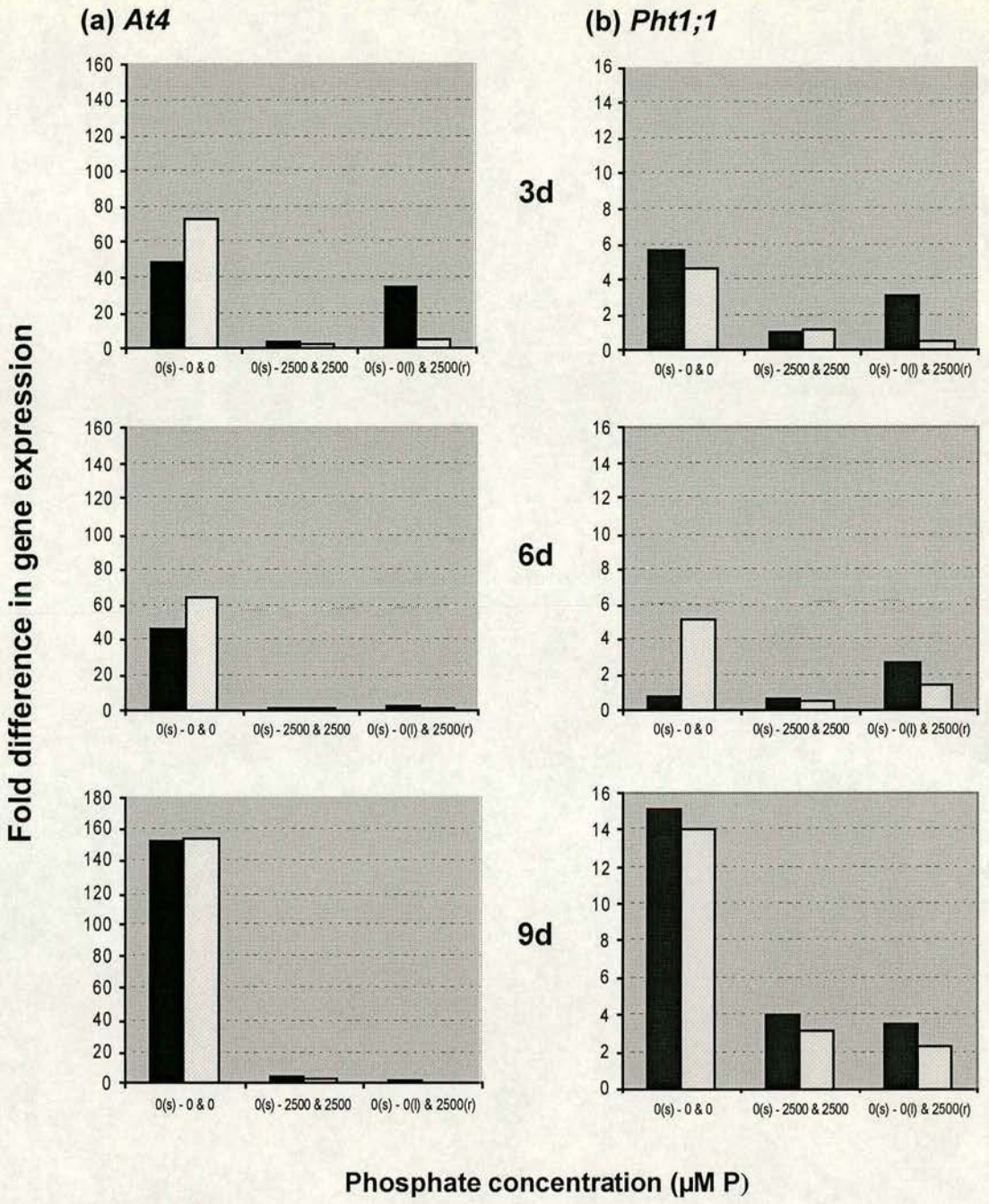


Figure 5.2: Split-root expression kinetics of a) *At4* and b) *Pht1;1* in Col 0 (wt) seedlings pre-grown in 125 µM Pi media and transferred to 3-compartment plates. Shoots were placed on no Pi solid media with roots separated into liquid media containing: no Pi in both sides (first 2 bars); high Pi in both sides (second 2 bars); and no Pi on the left side and high Pi on the right side (last 2 bars). Roots were sampled 3,6 and 9 days after transfer. Fold difference in expression, determined by Quantitative RT-PCR, is relative to expression in roots of a high Pi control seedling (3 d sample) and has been normalised for cDNA loading by comparing threshold cycles of amplification of the gene of interest to that of the constitutively expressed *eIF4A* gene. Data represent one of three independent repeat experiments.

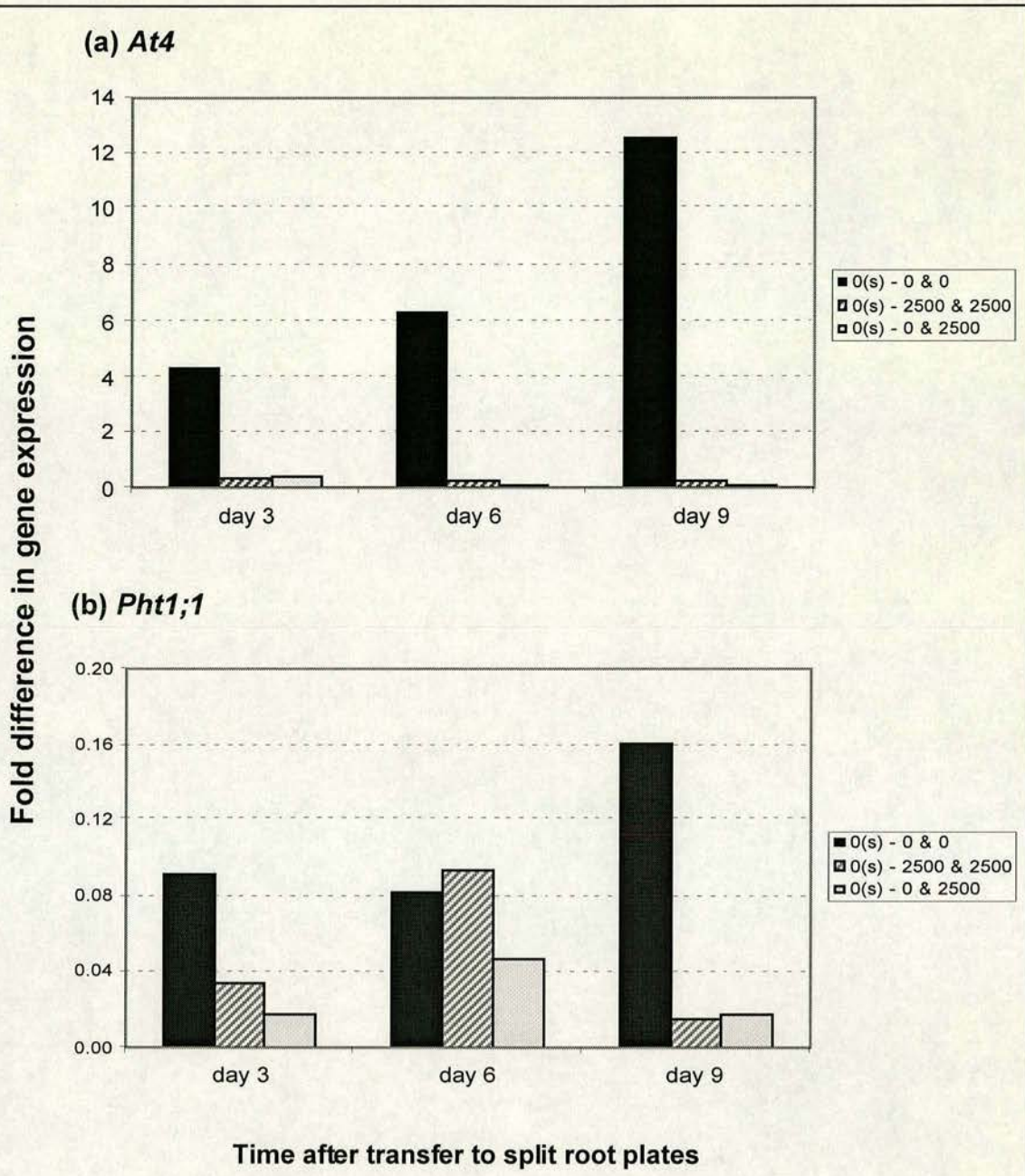


Figure 5.3: Shoot expression of a) *At4* and b) *Pht1;1* in Col0 (wt) split-root seedlings pre-grown in 125 µM Pi media then transferred to 3-compartment plates. Shoots were placed on solid no Pi media with roots separated into liquid media containing: no Pi in both sides (dark bars), 2.5 mM Pi in both sides (striped bars), and no Pi on one side and 2.5 mM Pi on the other (light bars). Shoots were sampled 3,6 and 9 days after transfer. Fold difference in expression, determined by Quantitative RT-PCR, is relative to expression in roots of a high Pi control seedling (3 d sample) and has been normalised for cDNA loading by comparing threshold cycles of amplification of the gene of interest to that of the constitutively expressed *eIF4A* gene. Data represent one of two independent repeat experiments.

is because shoot expression levels were calculated relative to the level of gene expression in roots, where these genes are predominantly expressed. *At4* expression increased with time in the shoots of starved control seedlings grown with both halves of the root system in Pi starvation media. The shoots of high Pi control seedlings displayed extremely low *At4* expression on all days tested. Interestingly, the shoots of split-root plants grown with one half of the root system in high Pi media and the other half in no Pi media showed very low *At4* expression after only 3 days, with expression levels similar to the high Pi control. This is despite still quite high expression in the starved half of the root system. Therefore, *At4* expression is down-regulated in both roots and shoots. Furthermore, down-regulation occurs earlier in shoot tissue.

Pht1;1 expression in the shoots of split root seedlings is again less clear. Shoot expression levels relative to root expression are extremely low. *Pht1;1* expression does increase with time in the shoots of starved control seedlings with both halves of the root system in no Pi media. However, expression seems to fluctuate in the shoots of control seedlings with both sides of the root system in high Pi. Nevertheless, by day 9 it is apparent that *Pht1;1* expression in shoots of seedlings with roots divided into high and low Pi is similar to that of the shoot expression of high Pi control seedlings. Therefore, *Pht1;1* expression is also systemically down-regulated in shoots.

5.4 Discussion

5.4.1 Systemic down-regulation of phosphate starvation response genes in *Arabidopsis*

The results presented in this chapter provide the first evidence for the systemic down-regulation of Pi starvation response genes in *Arabidopsis*. Previous reports of systemic signals controlling the expression Pi starvation response genes include the systemic down-regulation of high-affinity Pi transporters in tomato plants (Liu *et al.*, 1998a) and of the *Mt4* gene in the legume, *M. truncatula*. Long distance control of nitrate transporters has also been documented (Gansel *et al.*, 2001). The data reported here confirms that there is similar systemic control of the *Arabidopsis Pht1;1* high affinity Pi-transporter gene and of the *Arabidopsis Mt4* homologue, *At4*. Both genes were down-regulated in the starved half of a root system by a systemic signal derived in response to whole plant Pi status. Expression levels of *Pht1;1* were more variable and down-regulation was less distinct than for *At4*. This was probably because fold increases in *Pht1;1* expression in response to Pi starvation are relatively low (see chapter 3), creating a greater margin of error. It was also discovered that *MGD3* was systemically down-regulated in a manner similar to *At4* (see figure 6.3a). The *MGD3* gene has a physiological role and is involved in the synthesis of galactolipids to replace membrane phospholipids during Pi deprivation. Because of the diverse functions of the genes studied, it appears likely that most, if not all, Pi response genes are systemically down-regulated in response to plant Pi status.

Kinetic experiments revealed that down-regulation is only just apparent after 3 days (fig 5.2). Unfortunately, samples at earlier time-points yielded too little tissue for sufficient RNA extraction, therefore the exact timing of the beginning of down-regulation is not known. Nevertheless, it is clear that the intensity of the down-regulation signal increases with time and complete down-regulation becomes apparent after 6 days. It is also clear that the intensity of the signal is dependent on the overall plant Pi status. The magnitude of down-regulation in the starved half of the root system was less in plants with low Pi supplied to the other half of the root system, compared to those with high Pi supplied to the same half of the root system in (fig 5.1). This suggests that systemic signals are generated only once the Pi requirements of the plant are

satisfied. Therefore, it would be expected that, if lower Pi concentrations were supplied to one half of the root system, much later down-regulation of gene expression would occur in the other, starved half of the root system.

It is interesting that down-regulation of Pi starvation response genes also occurs in shoot tissue (fig 5.3). Both *At4* and *Pht1;1* were found to be expressed at substantially lower levels in shoot tissue. This is somewhat consistent with previous observations that *At4* and *Pht1;1* expression is root-specific (Burleigh and Harrison, 1999; Muchhal *et al.*, 1996). These previous examinations of shoot expression were performed by RT-PCR or Northern blot analysis and no transcripts were detected. However, the increased sensitivity of Quantitative RT-PCR used in my experiments indicate that there is a very low level of *At4* and *Pht1;1* expression in leaf tissue. There is also a small possibility that there was a lack of primer specificity, particularly in the case of *Pht1;1,* due to the highly conserved sequences of the Pht1 gene family. Whilst all efforts were employed to avoid this, the absence of Pht1 knockout mutants restricted efforts to test for amplification of other products. In any case, *At4* and *Pht1;1* expression in shoots was found to increase with Pi starvation and expression was also found to be down-regulated in split-root experiments. Down-regulation in shoots was complete after 3 days in split-root conditions. This is faster than the down-regulation of Pi response genes in the starved half of the root system. Down-regulation of Pi starvation response genes in shoots may occur in response to increases in intracellular shoot Pi, transported via the xylem stream from the high Pi side of the root system. In this scenario, a signal would then be generated in response to shoot Pi status to down-regulate gene expression in roots. Alternatively, the same systemic signal that down-regulates root gene expression may also mediate the down-regulation of shoot gene expression. Down-regulation would occur faster in leaf tissue as the signal is derived in the shoot and will take more time to reach root tissues.

The observation that gene expression is repressed in the high Pi half of the root system, prior to the down-regulation of expression in the starved half of the root system, provides further evidence that Pi starvation response genes are controlled both by local phosphate concentration and by systemic signals. Re-supply of phosphate to starved plants resulted in almost immediate recovery of Pi starvation response genes to basal expression levels (within 2 - 6 h) in section 3.3.6. It is expected that a similarly rapid response to local Pi availability would have occurred in the half of the root system

supplied with high Pi. Clearly, repression of Pi starvation response genes does not require a systemic down-regulation signal, if high external Pi is locally provided.

Coordinating Pi-starvation response genes at the level of the whole plant has its advantages. In the event of Pi limitation, plants induce Pi starvation responses to take up more Pi and to make Pi more available. This will continue until the necessary internal requirements are met. Once adequate amounts of Pi have been acquired, a down-regulation signal can then be generated to avoid expending energy on the maintenance of Pi starvation responses and to avoid additionally Pi accumulating to toxic levels. The problems caused by uncontrolled accumulation of Pi are highlighted by the *pho2* mutant which is unable to regulate shoot Pi concentrations. The symptoms of high Pi accumulation in this mutant include necrotic leaves and up to a 50% reduction in fresh weight (Delhaize and Randall, 1995). This illustrates the significance of regulating internal Pi concentrations and is indicative of the need for long-range signals to mediate this regulation.

5.4.2 Systemic signals

Phosphate itself seems the most likely candidate to act as a signal controlling the down-regulation of Pi starvation response genes. It is known that phosphate is re-mobilised from Pi-sufficient plant parts and is re-translocated to Pi-starved areas of the plant via the phloem (Schachtman *et al.*, 1998). The phosphate translocated from shoots to roots during Pi starvation may increase intracellular Pi levels of root cells, thus resulting in the down-regulation of Pi starvation response genes. Burleigh and Harrison (1999) tested this hypothesis by accompanying split-root gene expression studies with Pi measurements. From these experiments, it was discovered that Pi did not accumulate in the starved half of the root system, despite the presence of high levels of Pi in the shoot and in the half of the root system receiving high Pi fertiliser. This seems effectively to rule out Pi as the down-regulation signal. However, the level of Pi detected in the starved half of the root system was still slightly higher than the level of Pi found in the roots of starved control seedlings, grown with both halves of the root system in no Pi conditions. It is possible that this seemingly small difference may have an effect on Pi-starvation response gene expression. One other possibility is that organic Pi may be the systemic signal. Up to half of Pi detected in the phloem is in organic form (Bieleski *et al.*, 1973). The Pi determination method utilised by Burleigh and Harrison (1999) is

known to break down labile phosphate esters (Ames, 1966). Any difference in the concentration of these compounds between the two halves of the root system would have gone undetected in this P assay. However, as this phosphate assay was conducted on root tissue collected from plants grown in split-root conditions for 4 weeks, much of the phosphate re-translocated to the starved half of the root system would presumably have already been metabolised by cells and assimilated before this time.

The suggestion that organic P may be a systemic signal is perhaps questionable considering that the transporting form of phosphate in phloem is thought to be inorganic Pi, with organic P being mostly stationary in sieve elements (Bieleski *et al.*, 1973; Schachtman *et al.*, 1998). More comprehensive experiments are required to determine inorganic and organic P content in divided-root plants to investigate further this theory. The use of radio-labelled phosphate may prove a useful tool in determining the movement of organic and inorganic Pi fractions in split root plants.

The possibility of *At4* as a systemic signalling molecule in response to Pi availability was discussed in section 1.10.6. The small, putative encoded peptides, or the RNA molecules themselves, have been proposed as potential signalling candidates. As *At4* is itself down-regulated by a systemic signal, it is unlikely that *At4* is the systemic down-regulation signal unless the presence of *At4*, or its products, signals starvation.

Further discussion on phosphate signalling in *Arabidopsis* can be found in chapter 6 and a more general overview can be found in chapter 7 (Conclusion).

CHAPTER 6: DISSECTING SYSTEMIC SIGNALLING USING *ARABIDOPSIS* MUTANTS

6.1 Summary

Split-root experiments were conducted with various mutants to attempt to dissect phosphate-induced systemic signalling in *Arabidopsis*. Split-root experiments with *phr1-1* mutants ruled out the possibility that PHR1 may have an involvement in systemic signalling and adds further weight to the suggestion that it is involved in downstream Pi signalling. Delayed systemic down-regulation found in *pho1* mutants confirms that phosphate transfer to the shoot is necessary for the generation of a systemic signal. The absence of down-regulation in *pho2* mutants revealed that PHO2 is necessary for systemic signalling. Reduced shoot-root phosphate recycling in *pho2* mutants may account for this observation and implicates phosphate as the systemic signal. *35S::At4* seedlings displayed early down-regulation and is likely due to the delayed, reduced expression of Pi starvation response genes that occurs in *35S::At4* mutants. The cytokinin receptor mutant, *cre1-1*, was unaffected in systemic down-regulation and cytokinin application to wild-type split-root seedlings also did not alter down-regulation. This suggests that cytokinin is not the systemic signal, although further experiments are required to confirm this.

6.2 Introduction

In Chapter 5, it was established that systemic signals down-regulate Pi starvation response genes in *Arabidopsis* in response to internal phosphate status. By subjecting available *Arabidopsis* mutants to the same split-root treatments, it may be possible to uncover more about the nature of the systemic signal. The following candidates have previously been suggested as components of the phosphate-signalling pathway in *Arabidopsis*: a MYB transcription factor gene, *PHR1*; a Pi-response gene with unknown function, *At4*; the phytohormone, cytokinin; and phosphate itself (see section 1.10).

As yet, *PHR1* is the only gene that has been identified as a component of a Pi signalling pathway (see section 1.10.2). The expression of all Pi starvation response genes is defective in *phr1* mutants. The PHR1 protein binds to a specific sequence found in the promoter region of all Pi starvation response genes and is consequently considered

to be a downstream component in the Pi signalling pathway (Rubio *et al.*, 2001). Split-root experiments with the *phr1-1* mutant should determine whether PHR1 is necessary for systemic signalling.

The *Arabidopsis At4* gene of unknown function is highly upregulated during Pi starvation (Burleigh and Harrison, 1999). Its structure has prompted suggestions of a putative signalling role. The *At4* gene is composed of several, short, overlapping ORFs that have the potential to encode small signal peptides. Alternatively, the RNA itself may be the active signalling component. Here, seedlings transformed with the *At4* gene under the control of the 35S CaMV promoter, will be subjected to split-root treatment to determine whether systemic signalling is altered by the presence of constitutive *At4* expression.

Cytokinin as a potential Pi signalling molecule has been the subject of recent speculation (see section 1.10.7). The observed repression of Pi starvation response genes after cytokinin was applied to Pi limited *Arabidopsis*, and the reduced repression in cytokinin receptor mutants (*cre1*), confirmed cytokinin as a negative regulator of Pi starvation response genes (Martin *et al.*, 2000; Franco-Zorilla *et al.*, 2002). The possibility that cytokinin may be the systemic signal governing Pi response gene expresssion will be investigated by applying cytokinin to different halves of a wild type split-root system and by subjecting *cre1-1* mutants to split-root treatments.

Previous suggestions that phosphate re-cycling from shoot to root could act as the systemic signal regulating Pi starvation responses was previously ruled out by Burleigh and Harrison (1999). Yet, as discussed in section 5.3.2, the assay used to measure root Pi content may not reliably detect all forms of phosphate. Therefore, it should not be completely disregarded as a possibility. Split-root studies involving the *Arabidopsis* phosphate translocation mutants, *pho1* and *pho2,* may provide further information as to the role of Pi translocation in the regulation of Pi starvation response gene expression. PHO1 is thought to mediate Pi efflux from root stelar cells for loading into the xlyem stream. Plants with mutations in the *PHO1* gene have reduced Pi translocation to the shoot and are characteristically phosphate starved (see section 1.5.2). The *PHO2* gene has not yet been cloned but it is likely to play a role in phloem transport of Pi, or it may be involved in Pi sensing in cells. Mutations in result in the toxic accumulation of Pi in shoots and a reduction in Pi re-translocation from shoots to roots (see section 1.5.4).

Therefore, the aim of this chapter is to identify potential components of the systemic signalling pathway controlling global Pi starvation responses, by subjecting the relevant mutants to split-root treatment and observing the kinetics of systemic down-regulation in comparison to wild-type controls.

6.3 Results

To determine whether the kinetics of systemic down-regulation were altered in the chosen mutants, *Arabidopsis* Col0 wild-type and mutant seedlings were pre-grown in low phosphate (125 μM Pi) to limit the amount of Pi-storage, before being transferred to three-compartment plates. Their root systems were equally divided into two separate compartments containing variable phosphate concentration and grown for 3, 6 or 9 days. Quantitative RT-PCR analysis was performed on root tissue. Fold difference in gene expression is relative to expression in one side of the root system of a high Pi wild-type Col0 control plant (2.5 mM). Each split-root experiment was accompanied by a comparable wild-type split-root control experiment.

6.3.1 Split root experiments with *phr1-1* mutants

Figure 6.1a shows the kinetics of down-regulation in Col0 wild-type seedlings. Wild-type seedlings grown with one half of the root system in no Pi media and the other half of the root system in 2.5 mM Pi, displayed no down-regulation of *At4* expression in the starved half of the root system after 3 days. However, down-regulation was almost complete after 6 days and was maintained after 9 days in these conditions. The *phr1-1* mutant seedlings displayed similar down-regulation kinetics but with perhaps a slightly higher magnitude of down-regulation (fig 6.1b). It therefore appears that PHR1 is not a crucial component of the systemic signalling pathway that regulates Pi starvation responses in *Arabidopsis*. *At4* expression levels in the mutant were comparable with wild-type expression on all days and in both the control and treatment seedlings. This is surprising considering that *phr1-1* mutants have previously been found to display repressed expression of Pi starvation response genes.

6.3.2 Split root experiments with *pho1* mutants

Figure 6.1a illustrates the relevant wild-type control with which to compare *pho1* down-regulation kinetics (as above for *phr1-1*). *At4* expression in *pho1* mutants was similar to wild-type expression after 3 days in split-root conditions, with only slight down-regulation in the starved half of the root system of the variable Pi treated seedlings (fig 6.1c). However, down-regulation was still only slightly evident after 6 days, in contrast to the almost complete down-regulation found in the wild-type control.

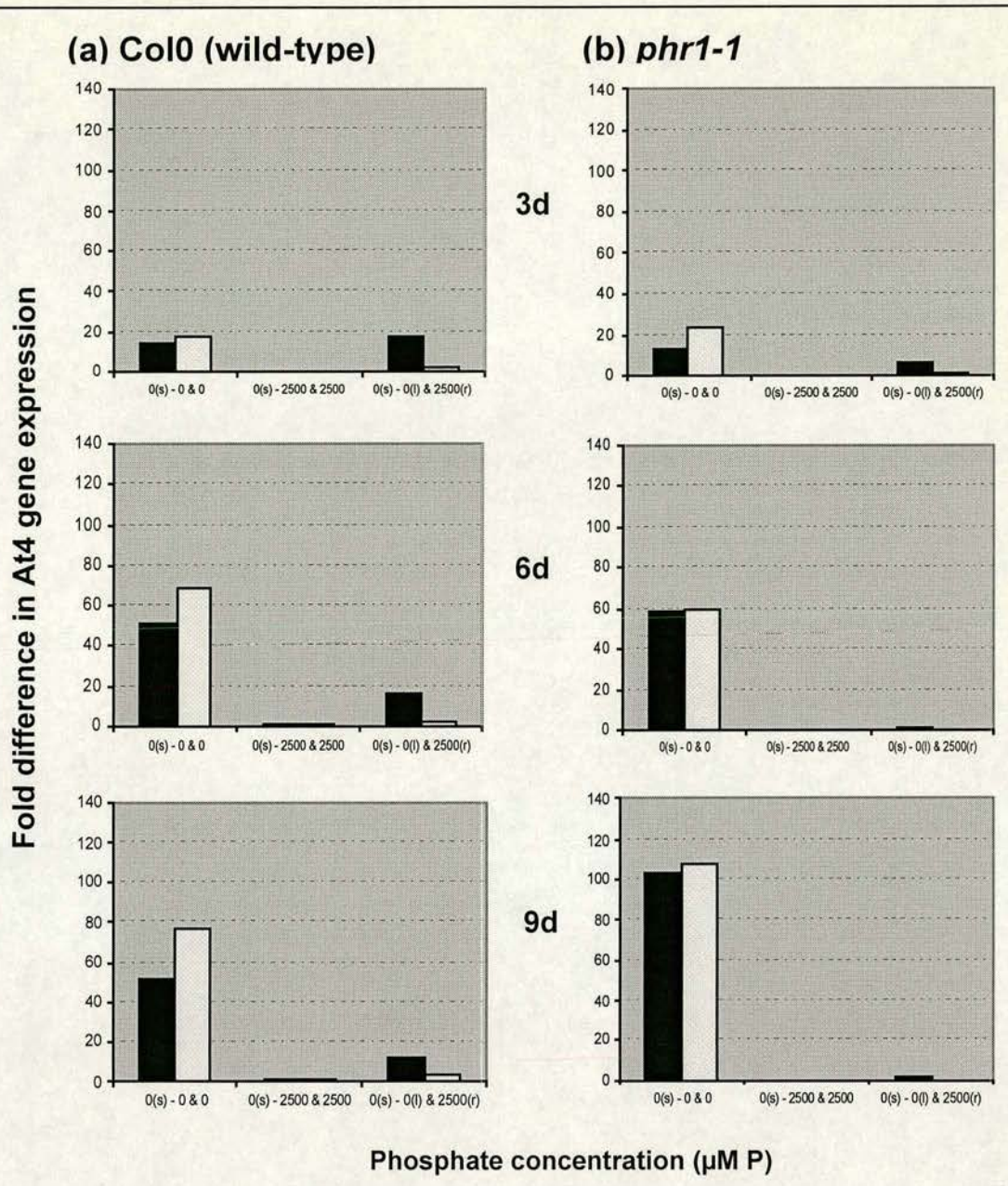


Figure 6.1: Split-root *At4* expression in a) Col0 (wt) and b) *phr1-1* mutants. Seedlings were pre-grown in 125 µM Pi media and then transferred to 3-compartment plates. Shoots were placed on no Pi solid media with roots separated into liquid media containing: no Pi in both sides (first 2 bars); high Pi in both sides (second 2 bars); and no Pi on the left side and high Pi on the right side (last 2 bars). Root tissue was sampled 3, 6 and 9 days after transfer. Fold difference in expression, determined by Quantitative RT-PCR, is relative to expression in roots of a high Pi Col0 control seedling (3 d sample) and has been normalised for cDNA loading by comparing threshold cycles of amplification of *At4* to that of the constitutively expressed *eIF4A* gene. Data represent one of two independent repeat experiments.

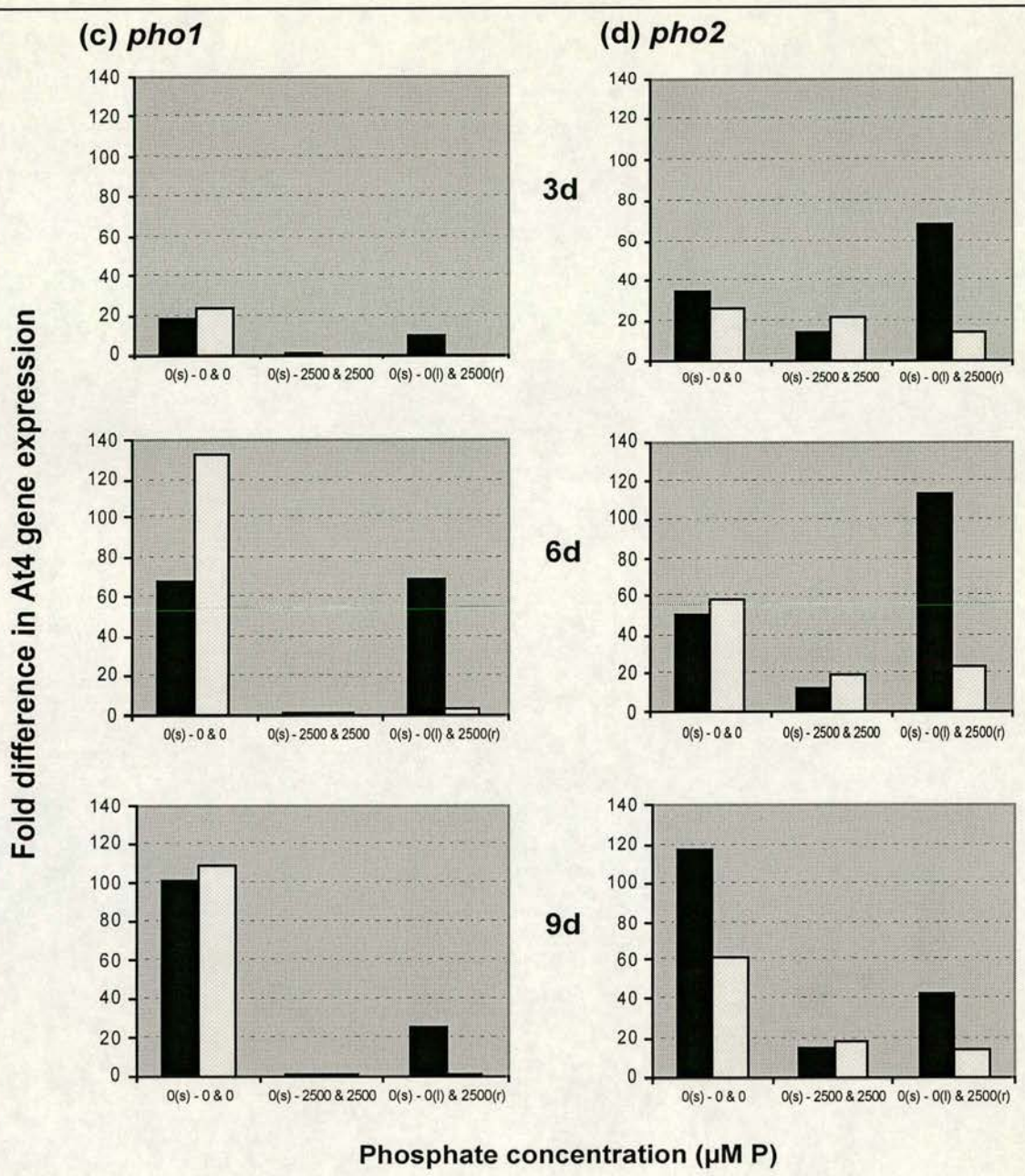


Figure 6.1 (continued): Split-root *At4* expression in c) *pho1* and d) *pho2* mutants. Seedlings were pre-grown in 125 µM Pi media and then transferred to 3-compartment plates. Shoots were placed on no Pi solid media with roots separated into liquid media containing: no Pi in both sides (first 2 bars); high Pi in both sides (second 2 bars); and no Pi on the left side and high Pi on the right side (last 2 bars). Root tissue was sampled 3, 6 and 9 days after transfer. Fold difference in expression, determined by Quantitative RT-PCR, is relative to expression in roots of a high Pi Col0 control seedling (3 d sample) and has been normalised for cDNA loading by comparing threshold cycles of amplification of *At4* to that of the constitutively expressed *eIF4A* gene. Data represent one of two independent repeat experiments.

Furthermore, *At4* expression was still higher than the wild type in the starved half of the root system after 9 days in split-root conditions, although expression had been further down-regulated since day 6. It seems that *pho1* mutants display slower kinetics of down-regulation than wild type plants.

6.3.3 Split root experiments with *pho2* mutants

Figure 6.1a illustrates the relevant wild-type control with which to compare *pho2* down-regulation kinetics (as above for *pho1* and *phr1-1*). *At4* expression in the starved half of the divided root system of variable Pi-treated *pho2* mutants was not down-regulated by day 3 or by day 6. Expression was somewhat down-regulated by day 9 but not to the same extent as wild-type down-regulation (Fig. 6.1d). It therefore appears that the systemic signal for down-regulation is disrupted in *pho2* mutants.

6.3.4 Split root experiments with *35S::At4* transgenics

Arabidopsis seedlings were transformed with a construct containing the *At4* gene under the control of the CaMV 35S promoter. A Northern blot of total RNA isolated from the roots of 8 seedlings homozygous for the construct and grown in high Pi (2.5 mM), was probed with ^{32}P-labelled *At4* cDNA and a ^{32}P-labelled *eIF 4A* probe as loading control (Fig. 6.2a). *At4* is massively over-expressed in all homozygous seedlings. Analysis of phosphorimaging data found that line no. 4 displayed the highest relative constitutive *At4* expression and this line was chosen for all further experiments.

Characterisation of *35S::At4* seedlings revealed that root growth responses to Pi availability were unaffected by high constitutive *At4* expression (figs 6.2b and c). On the other hand, gene expression was substantially affected. The kinetics of *Pht1;1* expression and MGD3 expression, in roots of *35S::At4* seedlings after transfer to Pi starvation, were compared with Col0 wt seedlings. The timing of expression of both genes was delayed in *35S::At4* seedlings. Col0 wt gene expression increased after 24 h, although the level of expression was higher at this time-point compared to previous kinetic experiments (see section 3.3.4). Col0 wt gene expression increased further with time, at levels similar to previous experiments. In contrast, expression began to increase only after 72 h in *35S::At4* seedlings (figs 6.2 d and e).

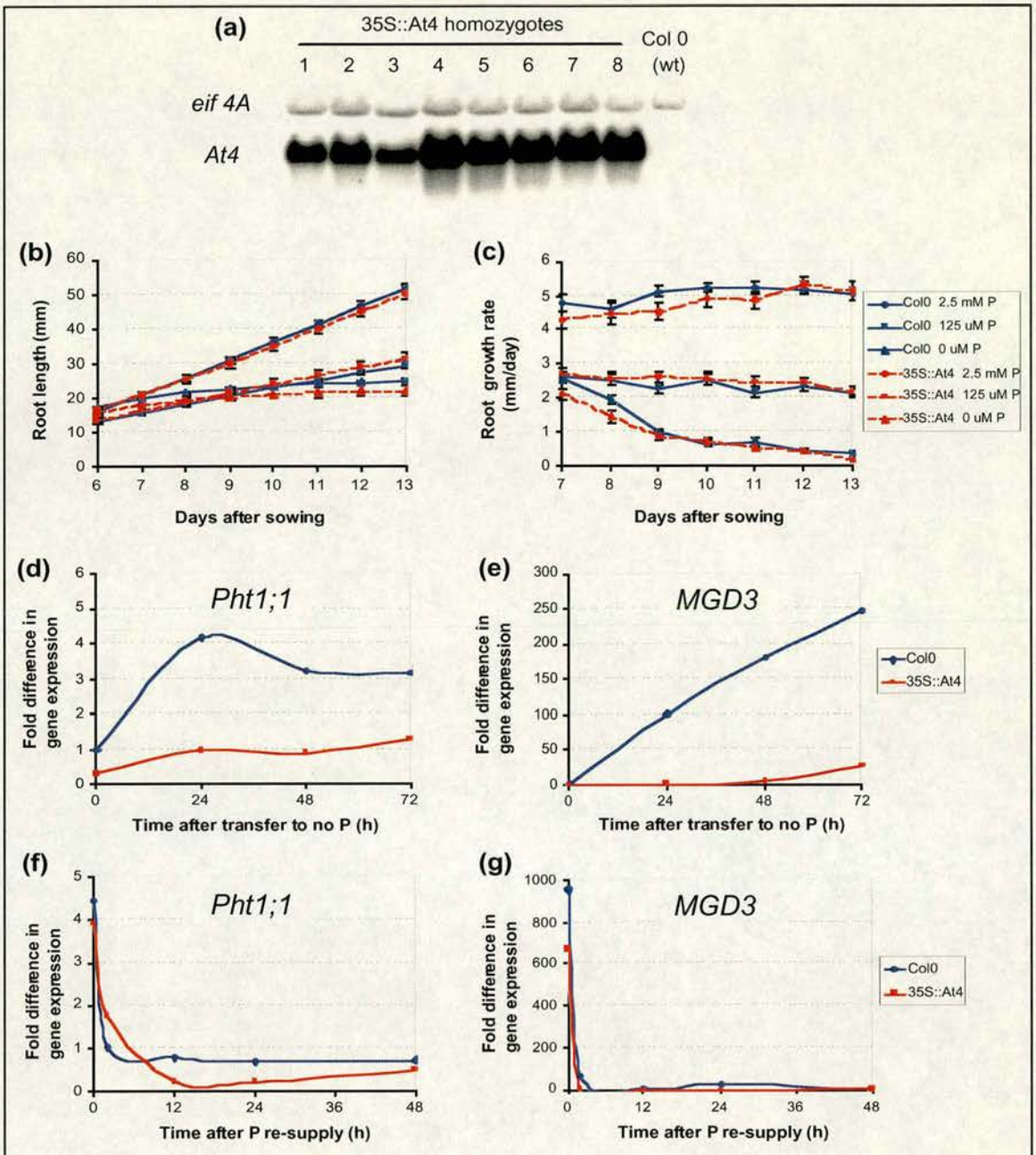


Figure 6.2: *35S::At4* characterisation a) Northern blot of total root RNA isolated from *35S::At4* homozygotes and Col0 (wt) seedlings grown in 2.5 mM Pi. Blot was probed with ^{32}P-labelled *At4* cDNA and a ^{32}P-labelled *eIF 4A* probe as loading control. b) Primary root length and c) primary root growth of *35S::At4* seedlings compared to Col0 (wt) seedlings, grown in vertical plates containing different Pi media d) *Pht1;1* and e) *MGD3* expression in roots of *35S::At4* and Col0 (wt) seedlings, transferred from 500 M Pi to no Pi (f) *Pht1;1* and (g) *MGD3* expression in roots of seedlings starved of Pi for 5 days and then re-supplied with 2.5 mM Pi. Fold difference in expression, determined by Quantitative RT-PCR, is relative to expression at t = 0 of starvation and has been normalised for cDNA loading by comparing threshold cycles of amplification of *At4* to that of the constitutively expressed *eIF4A* gene. Kinetic analysis data represent one of two independent repeats

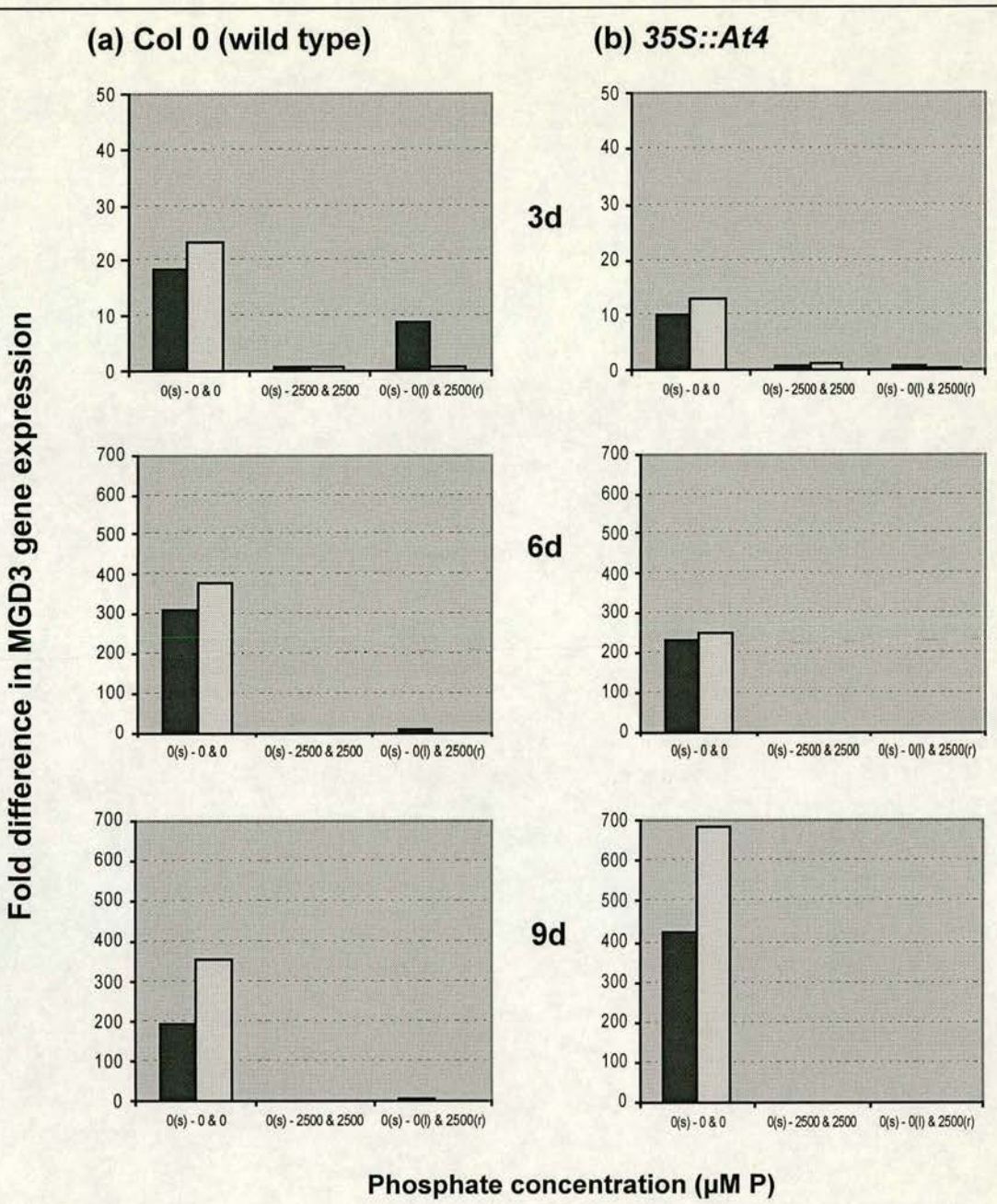


Figure 6.3: Split-root MGD3 expression in a) Col0 (wt) and b) *35S::At4* mutants. Seedlings were pre-grown in 125 µM Pi media and then transferred to 3-compartment plates. Shoots were placed on no Pi solid media with roots separated into liquid media containing: no Pi in both sides (first 2 bars); high Pi in both sides (second 2 bars); and no Pi on the left side and high Pi on the right side (last 2 bars). Root tissue was sampled 3, 6 and 9 days after transfer. Fold difference in expression, determined by Quantitative RT-PCR, is relative to expression in roots of a high Pi Col0 control seedling (3 d sample) and has been normalised for cDNA loading by comparing threshold cycles of amplification of MGD3 to that of the constitutively expressed eIF4A gene. Data represents one of two independent repeat experiments.

Five days after starvation conditions were imposed, expression of Pht1;1 and MGD3 in *35S::At4* seedlings had increased almost to wild-type expression levels (figs 6.2 f and g). At this stage, Pi was re-supplied. Recovery of gene expression to basal levels was similarly rapid in both *35S::At4* mutants and in wild-type plants. As *At4* is over-expressed in *35S::At4* seedlings, MGD3 gene expression was analysed in split-root experiments in place of *At4*. The kinetics of MGD3 down-regulation in wild-type split-root seedlings is similar to *At4* down-regulation kinetics (Fig. 6.3a), with some down-regulation after 3 days and complete down-regulation after 6 days. However, in the *35S::At4* mutant, down-regulation is already complete by 3 days.

6.3.5 Split root experiments with *cre-1* mutants and cytokinin application

The kinetics of down-regulation in split-root experiments with *cre-1* mutants (fig 6.4b) was compared to wild-type (fig 6.4a). Unfortunately, not enough RNA was isolated from day 9 *cre-1* samples, hence the missing graph. Also, time constraints did not allow for a repeat of this experiment. Therefore, conclusions drawn from these data have yet to be confirmed. However, initial indications from this experiment are that down-regulation is unaffected in *cre-1* mutants. Down-regulation was almost complete by 3 days in Col0 wt seedlings and was complete by day 6. The same response was observed in *cre-1* mutants.

Cytokinin was applied to either half of a wild-type split root system divided into high Pi (2.5 mM) and no Pi media. The wild-type control for this experiment is illustrated in figure 6.4a. When cytokinin was applied to the starved half of the root system, *At4* down-regulation was complete by day 3 (fig 6.4c). This occurred slightly earlier than for wild-type seedlings. When cytokinin was applied to the high Pi side of the root system, down-regulation was unaffected in the starved half of the root system (fig 6.4d).

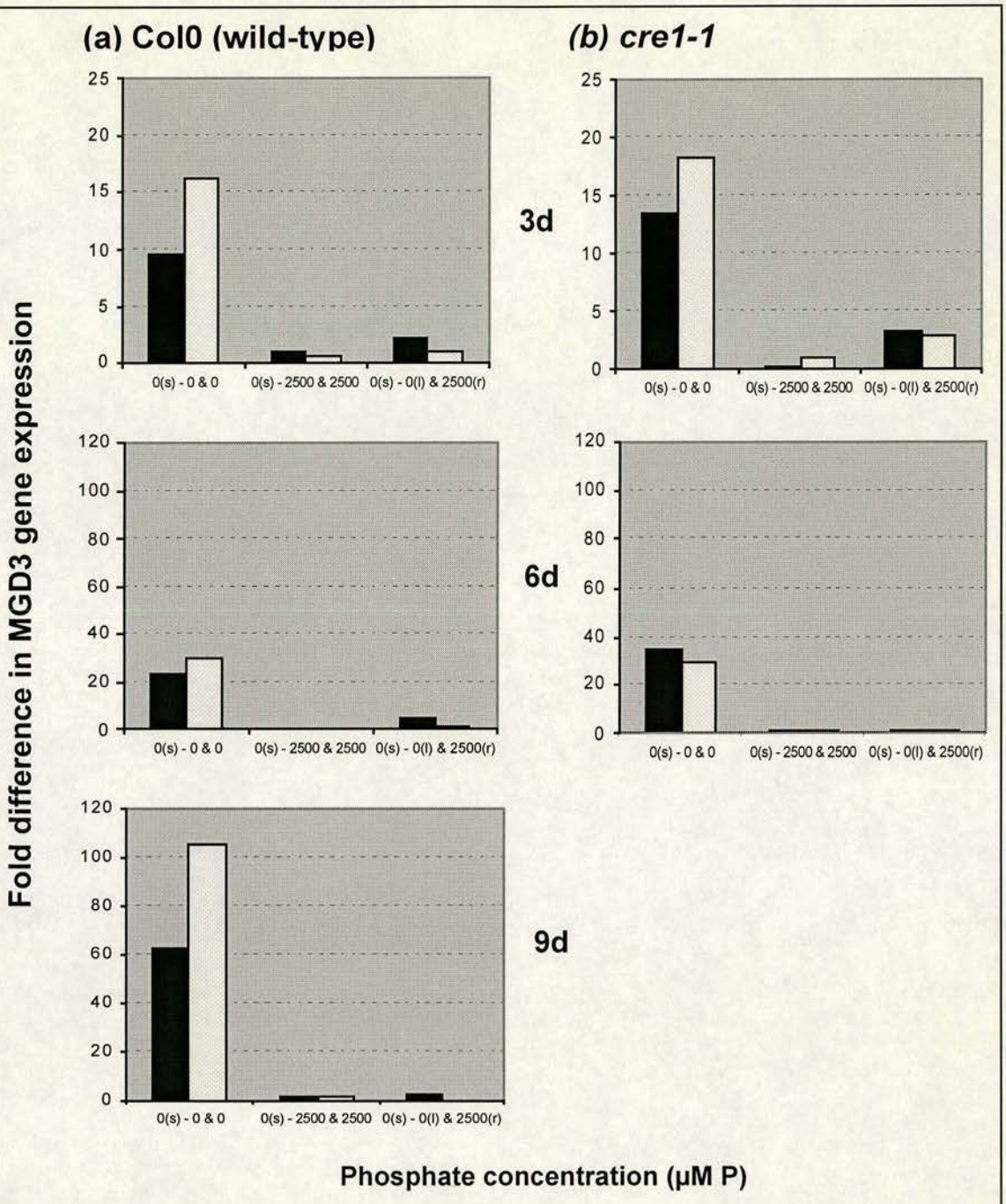


Figure 6.4: Split-root *At4* expression in a) Col0 (wt) and b) *cre1-1* mutants. Seedlings were pre-grown in 125 µM Pi media and then transferred to 3-compartment plates. Shoots were placed on no Pi solid media with roots separated into liquid media containing: no Pi in both sides (first 2 bars); high Pi in both sides (second 2 bars); and no Pi on the left side and high Pi on the right side (last 2 bars). Root tissue was sampled 3, 6 and 9 days after transfer. Fold difference in expression, determined by Quantitative RT-PCR, is relative to expression in roots of a high Pi Col0 control seedling (3 d sample) and has been normalised for cDNA loading by comparing threshold cycles of amplification of *At4* to that of the constitutively expressed *eIF4A* gene.

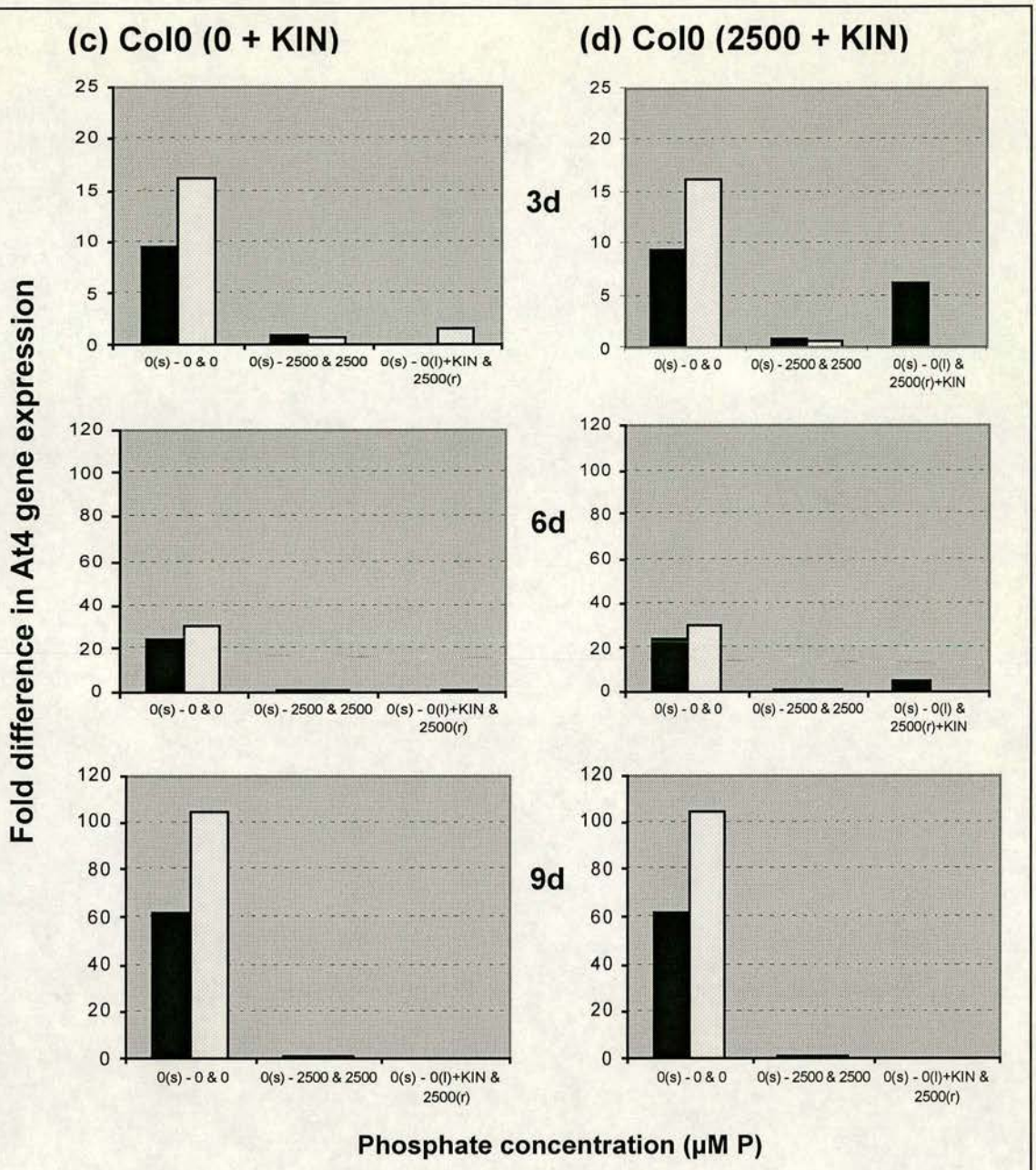


Figure 6.4 (continued): Split-root *At4* expression in Col0 (wt) seedlings with cytokinin applied to c) no Pi half of the root system and d) high Pi half of the root system. Seedlings were pre-grown in 125 µM Pi media then transferred to 3-compartment plates. Shoots were placed on no Pi solid media with roots separated into liquid media containing: no Pi in both sides (first 2 bars); high Pi in both sides (second 2 bars); no Pi on left side and high Pi on the right side +/- 10 µM KIN (last 2 bars). Root tissue was sampled 3, 6 and 9 days after transfer. Fold difference in expression, determined by Quantitative RT-PCR, is relative to expression in roots of a high Pi Col0 control (3 d sample) and has been normalised for cDNA loading by comparing threshold cycles of amplification of *At4* to that of the constitutively expressed *eIF4A* gene. Data represent one of two independent repeat experiments.

6.4 Discussion

6.4.1 PHR1 is not the systemic signal

As *At4* down-regulation was not altered in a *phr1-1* divided root system, PHR1 is unlikely to be the systemic signal. When compared to wild-type down-regulation kinetics in two repeat experiments, the only difference observed was a slightly greater down-regulation on day 3 of split-root treatment (Fig. 6.1b). *At4* expression is known to be reduced in *phr1-1* mutants (Rubio *et al.*, 2001). Therefore, earlier down-regulation in *phr1-1* mutants may occur because *At4* expression is constitutively lower. This means that *At4* expression should also have been repressed in the *phr1-1* starved split-root controls but this was not observed in the data presented here, nor in individual repeat experiments. Additional experiments were conducted to determine the expression kinetics of Pi starvation response genes in *phr1-1* mutants after transfer to starvation conditions. These experiments also revealed that the extent of repression of Pi starvation gene expression was less than that observed by Rubio *et al.* (2001). *At4* expression in *phr1-1* mutants was calculated as a fold difference relative to Col0 wild-type expression and was found to be approximately half wild-type expression levels (data not shown). This suggests that the repression of Pi starvation response genes in the *phr1-1* mutant is less severe than previously reported.

The observation that down-regulation is not perturbed in the *phr1-1* mutant reflects its putative role as a downstream component in the phosphate signalling pathway.

6.4.2 The systemic down-regulation signal is dependent on phosphate translocation to the shoot

Down-regulation of *At4* expression in the starved half of a split-root system was much slower in *pho1* mutants and was not truly apparent until after 9 days of split-root treatment (Fig. 6.1c). This response was observed in two individual repeat experiments. Translocation of Pi from roots to shoots is severely reduced in the *pho1* mutant (Poirier *et al.*, 1991). Thus, the delay in down-regulation observed in *pho1* mutants is most likely due to the reduction in Pi transfer from the high Pi half of the root system to the shoot. This confirms that the signal for down-regulation is dependent on shoot Pi status.

As *pho1* mutants are constitutively Pi starved, it may have been expected that *pho1* mutants would be Pi-starved prior to split-root treatment and that they subsequently should have displayed higher *At4* expression than Col0 wt seedlings. Instead, *At4* expression in control seedlings was similar to *At4* expression in wild-type controls. It should be noted that all seedlings were pre-grown in 125 μM Pi vertical plates with shoot tissue in direct contact with the media. As *pho1* mutants are defective only in xylem Pi loading, phosphate uptake by leaves remains unaffected. This means that the shoot content of *pho1* mutants may have been comparable with wild-type levels prior to split-root treatment. After transfer to split-root conditions, the only potential source of Pi for shoots is from root xylem transport as shoot tissue is placed on no Pi media.

When grown in minimal Pi media, only 0.9% of phosphate taken up by *pho1* mutants is transferred from roots to shoots, whereas 35% of Pi taken up by wild-type plants is transferred to the shoot (Poirier *et al.*, 1991). Consequently, it may seem surprising that enough phosphate is transported to the shoot to allow down-regulation to occur at all in *pho1* split-root mutants. However, it has previously been shown that discrepancies in shoot Pi content between *pho1* mutants and wild-type plants can be overcome through high exogenous Pi supply, possibly due to leakage of ions into the xylem, or by the activation of a separate low-affinity transporter in response to high Pi levels. Poirier *et al.* (1991) found that *pho1* shoot Pi levels were lower than wild-type levels when grown in 1 mM Pi media but were similar to wild-type levels at 5 mM Pi. The supply of 2.5 mM Pi to one half of the *pho1* root system in my split-root experiments was probably sufficient to force the transport of some Pi to the shoots and, by day 9, enough Pi had been transported to the shoot to initiate a down-regulation signal. Presumably, if more phosphate had been provided to that side of the root system, faster down-regulation would have been observed.

At4 expression was reduced in the roots of *pho1* mutants provided with high external Pi concentrations prior to the delayed systemic down-regulation of expression in the starved half of a split-root system. Therefore, the regulation of *At4* expression is not exclusively dependent on Pi translocation to the shoot, as was previously suggested by Burleigh and Harrison (1999). So, although *At4* expression is clearly systemically down-regulated, it is also under the control of local Pi availability. This observation supports the suggestion by Martin *et al.* (2000) that low shoot Pi status of *pho1* mutants

does not affect Pi starvation response gene expression in non-starved root cells. They reached this conclusion after observing that the expression of *AtIPS1*, an *Arabidopsis At4* homologue, was repressed in root epidermal and cortical cells of *pho1* mutants in a Pi-rich environment.

6.4.3 The systemic down-regulation signal is dependent on PHO2

The almost complete absence of systemic down-regulation in *pho2* mutants suggests that PHO2 is critical for the generation of a down-regulation signal (Fig. 6.1d). Characterisation of *pho2* mutants found that levels of phosphate were 2 to 5 fold greater in the shoots of *pho2* mutants compared to wild-type levels (Delhaize and Randall, 1995). As the signal for down-regulation is thought to be dependent on shoot Pi status, it was unexpected that the Pi-accumulating *pho2* mutants did not generate a systemic down-regulation signal. Unfortunately, PHO2 has yet to be cloned and its exact function is not known. It has been suggested that PHO2 may have a role in Pi sensing in cells or that it may function as a phloem transporter involved in the shoot to root transfer of phosphate cells. (Delhaize and Randall, 1995; Dong *et al.*, 1998). ^{32}P analysis revealed that the proportion of ^{32}P translocated in the phloem from shoots to roots in *pho2* mutants is less than half that found in the wild-type (Dong *et al.*, 1998). It has previously been suggested that re-cycled phosphate may be the signal for down-regulation (Forde, 2002). If this is the case, then reduced Pi re-allocation to roots in *pho2* mutants may account for the absence of down-regulation in the starved half of the root system. The idea that Pi recycled via the phloem acts as a signal to regulate Pi uptake by roots and to mediate Pi responses does stand to reason. It is known that less phosphate is re-translocated from shoots to roots during Pi limitation (Schachtmann *et al.*, 1998). The subsequent decline in root Pi concentration may be the trigger for the activation of Pi starvation responses in the root. Likewise, the reduced flow of Pi to roots may cause the increased root uptake of Pi observed in *pho2* mutants.

It is interesting to note that *At4* gene expression in high Pi split-root control treatments is higher in *pho2* roots than in the wild-type. This seems to counter previous conclusions that local high Pi availability represses the expression of Pi starvation response genes. However, as the down-regulation signal seems to override local starvation signals in the starved half of a split-root system, perhaps this is also true vice versa. If re-cycled Pi is the systemic signal, it is conceivable that the permanent

reduction in Pi re-cycling in *pho2* mutants partially over-rides local signals indicating high Pi status, hence the higher constitutive *At4* expression regardless of local Pi concentration. If this is the case, then organic P, rather than inorganic Pi, must be the systemic signal. The possibility of phosphate as a systemic signal was also discussed in section 5.3.2.

The high *At4* expression levels in the starved half of the *pho2* split-root system was unexpected. This does not occur in repeat experiments and, considering the variable expression levels observed even within control plants with both halves of the root system in starvation conditions (for example, starved *pho2* controls, day 9, Fig. 6.1d), it is unlikely to be significant.

6.4.4 The role of *At4* remains undetermined

The characterisation of *Arabidopsis 35S::At4* seedlings revealed interesting results. Constitutive *At4* expression did not affect root growth responses to phosphate availability. The recovery of gene expression after Pi was re-supplied to starved plants was also similar to wild-type recovery, indicating that local Pi signalling was unaffected in this mutant. However, the expression of Pi starvation response genes after transfer to starvation conditions was substantially delayed in *35S::At4* seedlings. This suggests possible disruption of internal Pi sensing. It may also indicate that *35S::At4* mutants have greater internal Pi stores. As discussed in Chapter 3, expression of Pi starvation response genes occurs later in seedlings with probable larger Pi stores. If *At4* has a role in Pi scavenging or acquisition, either directly or indirectly, then this is a plausible explanation. It would also account for the faster down-regulation of Pi starvation gene expression observed in *35S::At4* split-root seedlings (Fig. 6.3b) which, at first glance, seems at odds with the slower, later induction of gene expression seen in flask kinetic experiments (figs 6.2 d & e). Higher Pi status prior to split-root treatment would mean that down-regulation signals are already present and, consequently, Pi starvation gene expression would not be induced. Samples collected at earlier time-points in split-root experiments would prove useful in determining whether Pi starvation genes were induced in the starved half of the *35S::At4* split-root system.

As to whether *At4* is a systemic signal, the available evidence suggests not. In Chapter 5, it was discovered that *At4* is itself down-regulated, but *At4* could still be a systemic signal if its presence, or the presence of its products, results in the induction of

other Pi starvation response genes. If this is the case, constitutive *At4* expression in *35S::At4* plants should have resulted in enhanced expression of Pi starvation response genes. In fact, the reverse occurs and it therefore seems unlikely that *At4* is a systemic signal. In support of this, Rubio *et al.* (2001) found that *At4* expression is down-regulated in the *phr1-1* mutant along with all other Pi starvation response genes. Furthermore, they discovered that the PHR1-1 protein binds to a specific sequence found in the promoters of all Pi response genes, including *At4*. As PHR1-1 is considered to be downstream in the Pi signalling pathway, this would also suggest that *At4* is an end response to a signalling pathway, rather than a signal itself.

6.4.5 Cytokinin is likely not the systemic signal

Systemic down-regulation in *cre1-1* mutants was investigated because cytokinin is a potential systemic signal and cytokinin repression of Pi starvation responses is reduced in *cre1-1* mutants. (Martin *et al.*, 2000; Franco-Zorilla *et al.*, 2002). If cytokinin is the down-regulation signal, Pi starvation response genes should fail to be repressed in *cre1-1* split-roots. However, results from a preliminary split-root experiment found that the kinetics of down-regulation in *cre1-1* mutants is similar to wild-type down-regulation kinetics (Fig. 6.4b). Therefore, initial indications are that cytokinin is not the systemic signal. Further experiments are required before this can be confirmed. Firstly, the *cre1-1* mutants used in split-root experiments were in the Landsberg *erecta* background, yet they were compared to Columbia wild-type seedlings. Gene expression between ecotypes is has been found to differ quite significantly (Narang *et al.* 2000), therefore it cannot be ruled out as a possibility. Also, the loss of function strength of the *cre1-1* allele is unknown. Furthermore, a strong degree of redundancy is expected for the components of cytokinin signalling so it is possible that a putative systemic cytokinin signal could still have been perceived. It would therefore be prudent to test different *cre1* alleles in a Columbia background, and *cre1-4* in particular, as it likely represents a null allele (Franco-Zorilla *et al.*, 2002).

Application of 10 μM cytokinin to the starved half of a wild-type split-root system resulted in faster down-regulation of *At4* expression (Fig. 6.4c). This is consistent with previous observations that cytokinin represses Pi starvation response genes (Martin *et al.*, 2000). However, when cytokinin was applied to the high Pi half of the root system, the kinetics of down-regulation in the starved half of the root system

was unaltered (Fig. 6.4d). Nevertheless, this does not rule out cytokinin as a systemic signal. Perhaps a more revealing experiment would be the application of cytokinin to one half of a split-root system that has both halves divided into no Pi media. Whilst down-regulation would be expected in the half of the root system in direct contact with cytokinin, it would be interesting to observe whether systemic down-regulation occurs in the other (starved) half of the root system, in which down-regulation is not normally observed.

Therefore, cytokinin cannot be ruled out as a systemic signal until further detailed split-root analysis is performed.

See Chapter 7 (Discussion) for further insights into systemic signalling.

CHAPTER 7: CONCLUSION

It is evident from previous research, and the data presented here, that the response of plants to phosphate starvation is extremely complex. Responses are initiated at the level of growth, physiology and gene expression to reduce Pi demand, to conserve Pi reserves, to enhance Pi mobilisation and to increase access to Pi.

Measurements of primary root growth and root cortical cell size were used as indicators of growth response alterations to phosphate availability. Quantitative RT-PCR analysis was employed to determine the expression of Pi starvation response genes and of genes necessary for physiological alterations in response to Pi starvation. Quantitative differences in gene expression were found to vary with subtle changes in experimental conditions, therefore experiments were assessed depending on their qualitative repeatability. Kinetic experiments revealed that the timing and magnitude of all responses is dependent on pre-growth conditions i.e. on levels of internal Pi storage, with changes in physiology and gene expression preceding alterations in growth rate or morphology. Physiological alterations and changes in gene expression may occur first to reduce Pi demand and to increase Pi scavenging. This would ensure survival through transient periods of Pi starvation, whereas longer periods of Pi starvation would result in the commitment to irreversible and costly growth alterations. Primary root growth rate was found to be lower in Pi limited plants and was revealed to be partly due to parallel decreases in root cortical cell length. Molecular, physiological and growth responses were all enhanced by increased carbon availability. This suggests that metabolism, cellular Pi levels or Pi signalling may be altered by high C:P ratios. It also emphasises the need to consider the overall nutritional status of a plant as the balance of available nutrients is invariably tied to metabolism and cross-talk between different nutrient signalling pathways is also likely.

The main emphasis of my research was to uncover more about the nature of signalling in response to phosphate starvation. It was discovered that phosphate starvation response genes were very rapidly down-regulated when Pi was re-supplied to Pi starved plants. Expression of all genes recovered to basal level within 2 – 6 hours. This response was considered too fast to be under the control of systemic signals, indicating that local signals are responsible for the regulation of Pi starvation response

genes. The much slower induction of Pi starvation response genes that was observed when plants were transferred from adequate Pi media to starvation conditions, was probably due to the presence of Pi stores and to the re-translocation of phosphate to Pi-limited tissues. Therefore, it was conceivable that local signals were not initiated in these plants until the 'shielding effect' of Pi stores was diminished.

Split-root experiments showed for the first time in *Arabidopsis* that Pi starvation response genes are also under the control of a systemic signal. The delayed and reduced systemic down-regulation of Pi starvation response genes in *pho1* translocation mutants confirmed that the systemic signal was generated in response to internal shoot Pi status. The discovery that the same genes that were rapidly repressed by Pi re-supply were also systemically regulated, raised the possibility that Pi starvation response genes were under the control of both local and systemic signals. Experiments conducted with split-root plants supplied with different Pi concentrations indicated that the systemic down-regulation signal is initiated once the Pi requirements of the plant are satisfied. Also, the presence of the systemic signal seemed to override local signals indicating Pi limitation in the starved half of the root system. A model detailing proposed local and systemic signalling responses to phosphate in *Arabidopsis*, is provided in figure 7.1.

How might this model be reconciled with Pi starvation signalling in the natural environment? Soil nutrient availability is extremely heterogeneous and neighbouring sections of root will experience differing Pi availability. A section of root encountering a patch of soil low in Pi will initiate a rapid local induction of Pi starvation response genes to allow greater local uptake and mobilisation of P reserves. However, this will only occur in the absence of a down-regulation signal (i.e. when internal Pi stores are low). The local starvation signal will prevail until the overall Pi status of the plant is perceived to be adequate. In this instance, an antagonistic systemic signal will be generated to override local signals and subsequently down-regulate local Pi starvation responses. This would avoid expending energy on Pi starvation responses and would prevent the accumulation of toxic amounts of Pi.

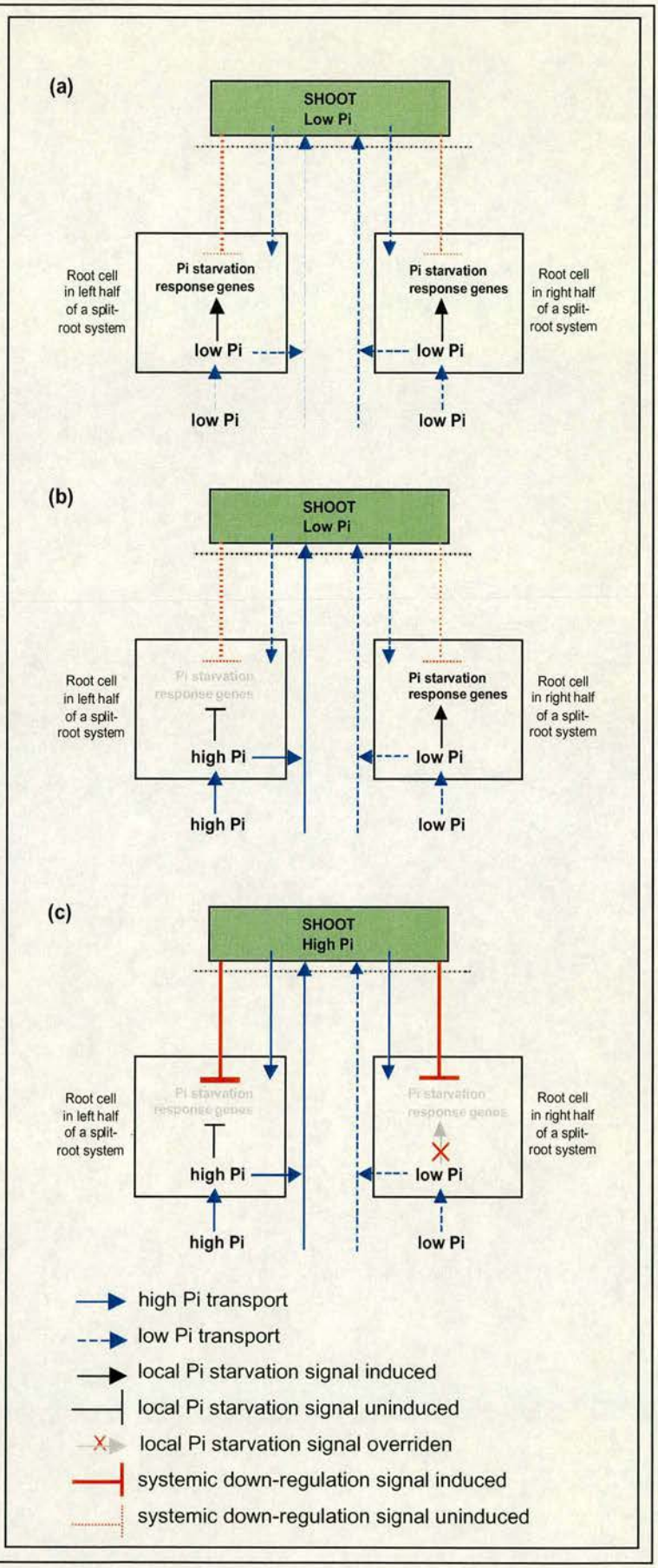
Split-root experiments conducted with mutants provided some insight into potential systemic signals. PHR1 was ruled out as a systemic signal because the down-regulation response was found to be normal in *phr1-1* mutants. Initial indications are

Figure 7.1: Proposed model for phosphate signalling in *Arabidopsis*

(a) Long-term Pi limitation means that shoot Pi stores are low. Phosphate re-translocation from shoots to roots is reduced. Local signals generated in response to low Pi availability induce Pi starvation response genes

(b) First few hours after supply of high Pi to the left half of the split-root system. Local Pi concentration in this half is high, resulting in the rapid local down-regulation of Pi starvation response genes. Pi is transported via xylem to shoot but has not accumulated to high Pi levels and Pi re-translocation is still low.

(c) Long-term supply of high Pi to the left half of the split-root system. Plant Pi requirements are satisfied A signal is generated to systemically down-regulate Pi starvation response genes, over-riding local signals indicating low local Pi status in the starved half of the root system. Pi re-translocation from shoots to roots increases.



that cytokinin is also not the signal, as normal down-regulation of Pi-starvation responses was observed in the cytokinin receptor mutant, *cre1-1*. However, further experiments are required to confirm this. The most intriguing insight came from split-root experiments with *pho2* mutants. These phosphate translocation mutants displayed a complete absence of down-regulation. PHO2 has no assigned function but it is thought to play a role in Pi sensing or in phloem transport of Pi. It is also known that Pi translocation is substantially reduced in *pho2* mutants (Dong et al., 1998). This makes it is tempting to suggest that Pi may be the systemic down-regulation signal. If phosphate were the signal, then the reduction in Pi re-translocation from shoots to roots in *pho2* mutants would explain the absence of down-regulation of local Pi starvation responses. Phosphate as the systemic signal also seems logical considering that Pi recycling to roots is reduced during Pi starvation in wild-type plants (Schachtman et al., 1998). This may well be the trigger for the induction of local Pi starvation responses. It would also explain why the induction of local Pi starvation responses is delayed in plants with high internal Pi stores, as Pi recycling likely continues until stores are depleted. In the model presented in figure 7.1, it is interesting to note that the re-cycling of phosphate from shoots to roots correlates with the presence of a systemic down-regulation signal. Yet the possibility of phosphate as a systemic signal has mostly been disregarded due to one observation by Burleigh and Harrison (1999) that Pi did not accumulate in the starved half of a *M.truncatula* split-root system displaying down-regulation of a Pi-starvation response gene. However, this phosphate assay had several shortcomings and these were discussed in section 5.3.2.

Phosphate as a systemic signal is a subject that should certainly be revisited. All forms of phosphate, including inorganic Pi, organic P and sugar phosphates, should all be considered as potential signalling candidates. Further experiments should be conducted with radiolabelled phosphate supplied to the starved half of a split-root system and the progression of phosphate re-translocation monitored. This should uncover more about phosphate re-cycling and its possible role as a starvation signal, and will hopefully contribute further to our understanding of plant phosphate starvation signalling and responses.

The model presented here for local and systemic phosphate signalling is reminiscent of the model for nitrate signalling suggested by Zhang and Forde (1998). They showed that a nitrate starvation-response gene, *ANR1* was required for lateral root

proliferation in response to local nitrate-rich soil patches. They also proposed the existence of an *ANR1*-independent shoot-derived systemic signal that suppresses lateral root elongation, depending on whole-plant ntirate status. It seems that this combination of local and systemic signals may be a common response by plants to heterogenous nutrient supply.

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